BIOLOGICAL ASSAY OF LACTOFLAVIN WITH CHICKS

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FOUR FIGURES

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In a previous communication (Lepkovsky and Jukes, '36 a) a diet for chicks was described, deficient in lactoflavin, but fairly adequate in other respects. Norris, Wilgus, Ringrose, Heiman and Heuser ('36) have recently summarized their investigations on 'vitamin G' in poultry feeding. They used chick growth as a method for 'vitamin G' assay, and state that "the dried pork liver used as a reference was found to contain approximately 100 micrograms of flavin per gram. In view of this, the reference liver was given a value of 100 chick units of vitamin G per gram. Hence a chick unit of vitamin G is approximately equal to 1 microgram of flavin." In the present investigation, diets similar to those used by Norris and co-workers in 'vitamin G' assay were found to be deficient in both flavin and the 'filtrate factor' (Lepkovsky and Jukes, '36 b). Dried pork liver is a rich source of these factors, both of which are strongly growth promoting (Lepkovsky and Jukes, '35). In order to assay lactoflavin by chick growth it is desirable to use a diet which will respond only to crystalline lactoflavin. An attempt to produce such a diet for the quantitative assay of lactoflavin in foodstuffs is described below.

EXPERIMENTAL

a. Demonstration of a double deficiency in a diet based on unheated corn meal, wheat middlings and purified casein

Chicks were placed at hatching on a basal diet similar to that used by Wilgus, Norris and Heuser ('35). It consisted of ground yellow corn, 55 parts; flour wheat middlings, '20; acid-washed casein, 18;[1] bone ash, 2; cottonseed oil (Wesson oil), 4; cod liver oil, 1; manganous sulfate, tetrahydrated, 0.03; hexane extract of alfalfa meal, equivalent to 1% of alfalfa meal (diet 169).

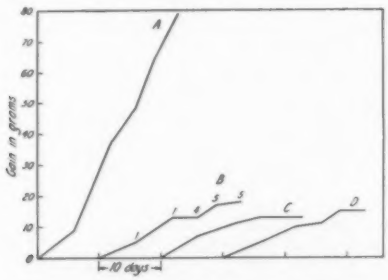

Fig. 1 Growth of chicks on diet 169 with various supplements. Six chicks were used in each group. The supplements fed to the chicks represented by curve A were lactoflavin and filtrate factor; curve B, lactoflavin; curve C, filtrate factor; curve D, basal diet (no supplement). The figures above curve B represent the number of chicks with dermatitis. No chicks in the other groups developed dermatitis.

The chicks were divided into four groups at the age of 14 days. The following supplements were used per 100 gm. of diet; group 1, 0.70 mg. of crystalline lactoflavin, prepared by Dr. S. Lepkovsky from whey adsorbate[2] as previously described (Lepkovsky, Popper and Evans, '35; Lepkovsky and Jukes, '36 a) plus 3 gm. of rice bran filtrate[2] (Lepkovsky, Jukes and Krause, '36); which had been treated three times with fuller's earth. The filtrate was found to be free from

[1] Supplied by the California Milk Products Co., Gustine, California.

[2] We are indebted to Vitab Products Inc., of San Francisco, for whey adsorbate and for the rice bran extract from which rice bran filtrate was prepared.

lactoflavin by testing it with rats. Group 2, 0.70 mg. of lactoflavin. Group 3, 3 gm. of rice bran filtrate. Group 4, no supplement. Group 1 grew rapidly and appeared normal in every way. Group 2 grew very slowly and developed typical 'chick dermatitis' (Lepkovsky, Jukes and Krause, '36). Groups 3 and 4 grew very slowly, but showed no symptoms of dermatitis. The results, illustrated in figure 1, made it evident that a diet for lactoflavin assay must be adequately supplemented with the filtrate factor.

b. Biological assay of lactoflavin with chicks

General methods in the care of chicks were previously described (Lepkovsky and Jukes, '35).[3] The birds were kept on ordinary mixed diet for 8 days following hatching, and were then placed on diet 96, low in lactoflavin, for from 6 to 10 days. At the end of this depletion period they were divided into experimental groups and placed on the test diets, which consisted of diet 96 plus the material to be assayed. Nine or ten chicks were used in each group. The starch and casein in the test diets were adjusted to maintain the nutritive ratio of the basal diet. An assay period of about 2 weeks was used.

Basal diet 96 had the following composition:

	parts
Yellow corn meal	30
Corn starch	23
Acid washed bran (Lepkovsky and Jukes, '36)	10
Washed casein [4]	22
Rice bran filtrate [5]	7
Sodium chloride	1
Ground limestone	1
Steamed bonemeal	1
Crude soybean oil [6]	3
Cod liver oil	2

Hexane extract of alfalfa meal, equivalent to 1% of alfalfa meal, evaporated on the diet.

[3] The F. E. Booth Co., San Francisco, kindly loaned battery brooders.
[4] See footnote 1, page 224.
[5] See footnote 2, page 224.
[6] Furnished by Allied Mills, Inc., of Peoria, Illinois.

Rice bran filtrate was prepared from rice bran extract as previously described (Lepkovsky, Jukes and Krause, '36), and was given five treatments with fuller's earth. The resulting preparation was found by assay to contain 20 units of the filtrate factor per gram (Jukes, '37). Seven per cent hence supplied an amount of the filtrate factor in excess of the amount required for maximal growth. The preparation was found to be free from lactoflavin by testing it with rats.

Acid-washed bran and soybean oil protected against gizzard erosions (Almquist and Stokstad, '37). The soybean

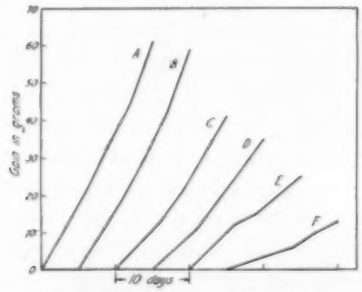

Fig. 2 Lactoflavin assay by chick growth, using diet 96, supplemented in the diet of the group represented by growth curve A with whey adsorbate 1, 3.0%; curve B, 2.0%; curve C, 1.0%; curve D, with crystalline lactoflavin, 0.28 mg. per 100 gm. of diet; curve E, 0.15 mg.; curve F, no supplement (basal diet). Nine chicks were used in each group.

oil was shown by chick tests with a 'purified diet' to furnish the anti-encephalomalacic factor of Goettsch and Pappenheimer ('36). Other details have been previously discussed (Lepkovsky and Jukes, '36 a).

The basal diet was tested by supplementing it with crystalline lactoflavin, with and without the addition of a vitamin B(B_1) concentrate prepared from rice bran (Lepkovsky and Jukes, '36 a). The vitamin B concentrate did not cause additional growth. A whey adsorbate [7] (Jukes, '37) was fed to some of the groups in order to standardize it against lactoflavin. This adsorbate will be referred to as 'whey adsorbate 1.' Some results are illustrated in figure 2 in which the growth

[7] See footnote 2, page 224.

curves show that the response to graded suboptimal doses of lactoflavin was recti-linear, since in this experiment an addition of 0.00015% of lactoflavin gave 12 gm., and 0.00028% gave 22 gm. increase in growth over the basal diet. Two per cent or 3% of whey adsorbate 1 both gave almost identical maximal growth responses of 46 and 48 gm. over the basal diet. The linear response indicated that 0.0006% of the sample of lactoflavin used was the level which would just suffice for maximal growth. The lactoflavin was tested with rats, and 5 micrograms daily produced a slightly better growth response than is required for 1 Bourquin and Sherman unit.

The results of a number of experiments indicated that an assay period of about 2 weeks with chicks gives practically the same results as a longer period. The following results serve as an example:

DIET	GAIN IN 13 DAYS	GAIN IN 25 DAYS	RATIO OF GAIN IN 25 DAYS TO GAIN IN 13 DAYS
Basal + 0.7% whey adsorbate 1	31	78	2.5
Basal + 2.0% whey adsorbate 1	68	150	2.2
Basal + 2.5% whey adsorbate 1	72	160	2.2
Basal + 3.0% whey adsorbate 1	65	152	2.3
Basal	11	25	2.3

The effect of prolonged depletion of chicks was tried. Ten chicks were kept on the basal diet from 7 to 91 days of age. During this time the average gain in weight was 90 gm. At the end of this period there were five survivors. Three of the birds which died showed 'curled-toe' paralysis similar to that described by Norris, Heuser, Wilgus and Ringrose ('30–'31). The survivors were very emaciated and relucant to stand. The average weight was 139 gm. They showed practically no comb growth. Their heads had a peculiar crow-like appearance due to disproportionately large beaks. The beaks were, however, smaller than is usual for birds of this age. Two of the birds were placed on a supplement of 3% of whey adsorbate 1. They responded dramatically. Their activity greatly increased within 3 days. In 33 days they gained an

average of 194 gm. Their combs grew quite noticeably. Both often exhibited a rapid tremor of the head and neck. The birds remaining on the basal diet all died within 12 days. Similar results were obtained by injecting crystalline lactoflavin (fig. 3). Figure 4 shows the appearance of a normal bird in contrast.

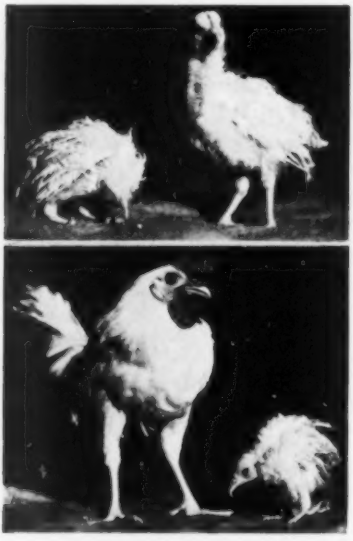

Fig. 3 The two chicks above were placed on basal diet 96 when 7 days old. At 97 days, the bird at the right received a single intramuscular injection of 1 mg. of crystalline lactoflavin dissolved in 2 cc. of 0.9% sodium chloride solution. Seventeen days later, when the picture was taken, its weight had increased from 101 to 190 gm. During the same period, the uninjected bird on the left had increased in weight from 89 to 96 gm.

Fig. 4 One hundred and fourteen-day-old chicks raised respectively on ordinary mixed diet and on diet 96.

Diet 96 was used for the assay of lactoflavin in feeding stuffs. A positive control diet consisting of diet 96 plus 2% of whey adsorbate 1 was employed, and the results were given an arithmetical treatment similar to that used in calculating the 'filtrate factor value' and 'filtrate factor unit' (Jukes and

Lepkovsky, '36; Jukes, '37). Table 1 summarizes various assays in terms of 'chick units of lactoflavin.' One chick unit is one-tenth of the amount which will just provide for maximal growth when fed daily to a chick during the assay period

TABLE 1

Distribution of lactoflavin in feeding stuffs. One chick unit is equivalent to about 1 modified Bourquin and Sherman rat unit

MATERIAL	PER CENT FED IN BASAL RATION	GROWTH RESPONSE	GROWTH ON POSITIVE CONTROL DIET	GROWTH ON BASAL DIET	LACTOFLAVIN CONTENT IN UNITS PER GRAM
Brewers' yeast 1	2	25	58	12	14
Young alfalfa shoots, dried at 40° (fall)	8 (dried basis)	47	55	15	10 (dried basis) 2.0 (fresh basis)
Lawn clippings dried at 40° (fall)	8 (dried basis)	41	55	15	8 (dried basis) 2.0 (fresh basis)
Dried skim milk	3	30	68	13	10
	5	33	48	20	10
	8	42	58	12	8
Alfalfa leaf meal	8	35	58	12	6
Dehydrated alfalfa meal	8	41	58	12	8
Wheat germ	10	28	55	15	3.1
	10	34	67	18	3.3
Soy bean meal	10	23	58	12	2.4
Sesame meal	10	20	58	12	1.7
Hempseed meal	10	20	55	15	1.2
Peanut meal	10	17	58	12	1.1
Cane molasses	5	10	58	12	....
Whey adsorbate 1[1]	1	41	60	13	60
	1	37	53	9	64
	1	39	70	10	48
	1	57	75	18	68

[1] Sixteen and six-tenths milligrams fed daily to rats resulted in an average gain of 20 gm. in 4 weeks, i.e., the adsorbate supplied about 60 modified Bourquin and Sherman units (Dimick, Smith and Davis, unpublished) per gram. We are indebted to Mrs. M. I. Dimick of Vitab Products, Inc., for the rat assay.

under the conditions employed. The assay period lasted from the end of the second to the end of the fourth week of the chicks' age, hence the average age was 3 weeks. The average daily food consumption when diet 96 was adequately or nearly adequately supplemented with lactoflavin was about 10 gm.

Hence, as in the case of the 'filtrate factor unit' (Jukes, '37), the number of units of lactoflavin per gram of a supplement was calculated by dividing the net gain on the supplemented diet by the net gain on the positive control diet multiplied by the amount of the supplement in 1 gm. of the supplemented diet. One chick unit was approximately equal to 6 micrograms of the sample of crystalline lactoflavin used. The degree of purity of the sample is not known. One chick unit was approximately equal to a modified Bourquin and Sherman unit of 'vitamin G,' determined by rat growth on a diet deficient only in lactoflavin (Dimick, Smith and Davis, unpublished).

There is a marked difference in distribution between lactoflavin and the filtrate factor in common feeding stuffs. Filtrate factor assays (Jukes and Lepkovsky, '36; Jukes, '37) of some of the materials whose lactoflavin contents were also determined gave the following results.

MATERIAL	CONTENT IN UNITS PER GRAM OF	
	Filtrate factor	Lactoflavin
Brewers' yeast	13.0	14.0
Dried young alfalfa shoots	1.0	9.0
Dried lawn clippings	<0.2	8.0
Dried skim milk	3.0	9.0
Alfalfa leaf meal	1.3	6.0
Dehydrated alfalfa meal	3.0	8.0
Peanut meal	3.5	1.1
Cane molasses	6.0	Not detected
Whey adsorbate 1	<0.2	60.0

DISCUSSION

Wilgus, Norris and Heuser ('35) state that their basal diet "was seldom deficient in the antipellagric phase, and then so slightly that growth was not retarded." In the present investigation it was found that a similar basal diet was deficient in the 'chick antipellagric' (filtrate) factor to an extent which greatly retarded growth. The lack of agreement is probably due to differences is the filtrate factor content of the natural feeding stuffs used in the basal diets in the respective investigations. Birds on diet 169 did not show symptoms of dermatitis unless lactoflavin was added. Previous observations

(Elvehjem and Koehn, '35; Lepkovsky and Jukes, '35; Lepkovsky, Jukes and Krause, '36) have shown that dermatitis due to a deficiency of one member of the vitamin G complex is much more pronounced when the other members are supplied in ample amounts. Growth promotion, of course, is the function of all definitely characterized members of the vitamin B complex, and a mild deficiency of the 'filtrate factor' often results in retardation of growth without the appearance of dermatitis in chicks (Jukes, '37).

A short assay period minimizes the possibility of an outbreak of cannibalism which often occurs when chicks are confined to battery brooders for a long time. Depleted chicks and balanced groups must be used to obtain accurate results in a short-period assay. Balanced groups were selected at the end of the depletion period by dividing all the chicks into ten weight classes and taking one chick from each weight class to form a group.

The results in this investigation indicated that certain commonly used 'vitamin G' supplements, such as greens, alfalfa meal and dried milk are, in terms of similarly computed chick growth units, much richer sources of lactoflavin than of the filtrate factor. In ordinary poultry rations, this difference is compensated to some extent by the fact that the cereal grains and their by-products furnish appreciable amounts of the filtrate factor, although they are poor sources of lactoflavin.

Units of vitamins obtained by assay with rats are often based upon grams of gain per week. The chick unit used in this investigation and in previous work (Jukes and Lepkovsky, '36) is based upon percentage of maximal net growth obtainable, measured by the gain of a positive control group minus the gain of a group on the basal diet. The use of net growth rather than total growth compensates for 1) small amounts of the vitamin in the basal diet, inevitably present, since successful chick diets always include unpurified natural feeding stuffs, 2) vitamin reserves in the body of the chick. The use of a positive control diet minimizes effect of variations in growth rate in different hatches of chicks. There are great variations in growth rate at different times of the year in

chicks of the same strain. The following results indicate the extent of the variations which were encountered in the present investigation:

Experiment no.	1	2	3	4	5	6	7	8	9	10
Gain in 14 days on		68	65						55	
positive control diet	53	66	69	50	60	53	49	43	57	55

Gains of the other groups in each experiment were never greater than the gain of the positive control group or groups. In experiments in which the positive control group grew comparatively slowly, the growth of the assay groups was apparently proportionately slower.

The results of the experiment on prolonged depletion were in harmony with the observations of Bethke, Record and Wilder ('36) who found that pure flavin preparations prevented the occurrence of a leg disorder (Norris and co-workers, '30–'31; Bethke and co-workers, '30–'31).

SUMMARY

1. A diet similar to that used in 'vitamin G' assays (Norris and co-workers, '36) was found to be deficient in both lactoflavin and the filtrate factor. Addition of crystalline lactoflavin to the diet appeared to provoke dermatitis in chicks.

2. A basal diet for chicks is described which was used in the biological assay of lactoflavin by growth. Maximal growth under the conditions of assay required the addition of about 0.60 mg. of a crystalline preparation of lactoflavin to 100 gm. of the diet. The exact purity of the lactoflavin used was not known. The growth response to lower levels was roughly proportional to the amount of lactoflavin which was fed.

3. Symptoms of prolonged lactoflavin deficiency in chicks are described.

4. The lactoflavin content of some feeding stuffs is reported in terms of a chick unit which was based on chick growth, and was equivalent to one-tenth of the daily amount which will just provide for maximal growth under the conditions described. The unit was approximately equivalent to a modified Bourquin and Sherman unit of 'vitamin G.' Under the con-

ditions of the assay, the requirement of the chick for maximal growth was about 100 units per 100 gm. of diet.

5. Attention is drawn to the differences in distribution of lactoflavin and the filtrate factor is certain feeding stuffs.

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ASSAY PROCEDURE FOR VITAMIN K (ANTI-HEMORRHAGIC VITAMIN)

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That the absence of vitamin K in the diet of the chick leads to deficient blood clotting power, hemorrhages and anemia, has been shown in a number of papers (Dam, '35 a, '35 b; Almquist and Stokstad, '35 a, '35 b). Schonheyder ('36) has described the method of assay used by Dam and Schonheyder ('36). In brief, this method consists of rearing chicks on a normal diet for 2 weeks, then on a vitamin K deficient diet for at least 3 weeks at which time all animals are assumed to be depleted of vitamin K. Blood samples are taken from a carotid artery by cannula and centrifuged. The resulting plasma is diluted with Ringer's solution and the amount of a clotting agent necessary to cause coagulation of the plasma in approximately normal time is determined. Substances to be tested for vitamin K are then given by capsule to birds of the same group and these birds are tested for clotting time after several days. It appears to us that this method is unnecessarily complicated for practical purposes, lengthy from the standpoint of time required in obtaining depleted birds, and open to errors due to the introduction of a new factor, the clotting agent.

The preventive method used in our laboratory is based upon the occurrence of hemorrhage and upon simple measurement of blood clotting time. Striking differences in regard to hemorrhages and clotting time in various groups have frequently been obtained before the chicks were 2 weeks old. Some of

the precautions necessary in producing distinct deficiency at such an early date have already been mentioned (Almquist and Stokstad, '36).

The present paper deals with the assay procedure in detail and its application to the testing of various substances.

METHOD

Groups of newly hatched chicks were placed in metal, wire floored electrically heated battery brooders and given diet E as described in a previous paper (Almquist and Stokstad, '36). The substance to be tested was intimately mixed with the diet at suitable levels, in the case of solids by fine grinding and mixing, in the case of fat-soluble substances by solution in hexane and mixing the hexane solution thoroughly in the diet. The hexane soon evaporated leaving the substance uniformly dispersed in the diet.

Blood samples were taken from chicks by puncture of a main wing vein in such manner as to avoid cutting of muscular tissue and by allowing about 0.2 cc. of blood to fall into a clean glass vial. The vial was placed at once in a water thermostat at 38.5°C. and shaken by a constant speed mechanism. When the blood was no longer able to flow as the tube was shaken, it was considered to have clotted. The time of withdrawal of the blood sample and the time of clotting were recorded. The difference, in minutes, was taken at the clotting time. When this time was greater than 30 minutes, the blood sample was considered negative.

Since anemia had been observed as a portion of the general syndrome, it seemed advisable to include a study of hemoglobin levels as a possible aid in the procedure for determining adequacy of vitamin K supplements. Hemoglobin was measured by the Wu modification of the acid hematin method. The standard was a sample of chicken blood in which the hemoglobin level was determined by the oxygen capacity method. Comparison of the blood samples with the standard was made by a colorimeter.

RESULTS AND DISCUSSION

Results obtained during an assay of a vitamin K concentrate are given in table 1. The information in table 1 leads to several conclusions of interest.

1. Comparison of group 5 with group 6 shows that group 5 was somewhat deficient in vitamin K, giving evidence of prolonged clotting time and hemorrhage. Group 6 receiving 20% more vitamin K was not deficient. The procedure may therefore be regarded as capable of detecting slight deficiencies as well as complete deficiencies.

2. Prolonged clotting time was found in the first six groups at 1 week of age and was especially prevalent in the first four groups. This fact indicates rapid exhaustion of the vitamin K reserves of the newly hatched chick. It also indicates that the observations of clotting time may give significant results even earlier than observations of hemorrhage.

3. While prolonged clotting time was found at 1 week in deficient groups, hemorrhages were also noted in all of these groups at the same or a later date. The first two groups would probably have shown a high incidence of spontaneous hemorrhage had not most of the chicks died from bleeding as a result of the taking of blood samples. Thus the observation of hemorrhage leads ultimately to the same result as measurement of clotting time.

4. The average hemoglobin values are of special interest as they show that at an age of 1 week, when prolonged clotting time was found in a majority of the chicks in the first four groups but hemorrhage had affected only two of these chicks, the hemoglobin values were not appreciably different from those of the last three groups and particularly the last or non-deficient group. This evidence is in agreement with the statement of Schonheyder ('35) that deficient blood shows no abnormalities other than lack of clotting power. Our data show explicitly that avitaminosis K does not lead directly to anemia. The anemia reported in vitamin K deficient chicks (Dam and Schonheyder, '34; Holst and Halbrook, '33) must be regarded entirely as a consequence of hemorrhage.

TABLE 1

Relationship of blood clotting time, hemoglobin level and hemorrhage (eight chicks in each group)

GROUP NUMBER AND VITAMIN K CONCENTRATE, MILLIGRAMS PER KILOGRAM OF DIET	CLOTTING TIME TO NEAREST MINUTE, AT AGE OF			AVERAGE HEMOGLOBIN LEVEL, GRAMS PER 100 CC., AT AGE OF			HEMORRHAGIC SYMPTOMS, NUMBER OF CHICKS AT AGE OF			DIED FROM BLEEDING, NUMBER OF CHICKS AT AGE OF		
	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks	1 week	2 weeks	3 weeks
0	15, 12 (6)[1]	(4)[1]	.....	7.1	5.0	...	0	1	..	4	2	..
1	12, 17 (6)	6 (1)	.....	7.6	...	...	0	3	..	4	2	..
2	9, 11 (6)	4, 5, 4, 7, 3 (2)	25, 3, 17, 3 (2)	7.2	5.9	6.8	1	3	0	1	1	2
3	(8)	23, 24, 27, 17 (2)	4, 2, 2, 8, 2	7.3	6.2	7.5	1	2	0	2	1	0
4	9, 7, 10, 8, 8 (3)	25, 3, 4, 6 (2)	11, 7, 4, 5, 3	7.2	5.8	7.0	0	1	0	1	1	0
5	3, 13, 10, 4, 4, 4, 10 (1)	28, 6, 2, 5, 8 (2)	3, 3, 16, 2, 3, 21	6.6	4.9	6.6	0	2	0	1	1	0
6	10, 2, 2, 1, 6, 2, 7, 3	2, 3, 1, 1, 3, 2, 1, 2	8, 4, 3, 2, 4, 3 3, 5	6.8	5.7	7.7	0	0	0	0	0	0

[1] Figures in parentheses refer to number of birds in which blood clotting time was greater than 30 minutes.

The lower hemoglobin levels in the second week are in agreement with the report of Harmon ('36) that chicks on normal diets show a minimum hemoglobin level at this age. To some extent, the hemoglobin values of the deficient groups may have been influenced by hemorrhage, however, they were not appreciably different from that of the last or non-deficient group. The hemoglobin values showed a rise at the third week, as would be expected (Harmon, '36). It is evident that determination of hemoglobin level does not constitute a necessary part of a vitamin K assay.

TABLE 2

Effect of soybean oil on blood clotting time of chicks approximately 2 weeks old

SUPPLEMENT	NUMBER OF CHICKS	HEMORRHAGIC SYMPTOMS NUMBER OF BIRDS	AVERAGE CLOTTING TIME IN MINUTES
None. Negative group control	10	4	12 (7)[1]
Hexane extract of alfalfa equivalent to 1%. Positive control group	10	0	1.7
Soybean oil 1%	7	0	15.6
Soybean oil, 2%	10	0	3.7
Soybean oil, 5%	8	0	1.3

[1] Figure in parentheses refers to number of birds in which blood clotting time was greater than 30 minutes.

5. The data suggest that at the third week the surviving chicks in groups three, four and five were spontaneously recovering since clotting time was restored toward normal. This may have been due to increased food intake, or a lowering of requirement for vitamin K as the birds became older. We have noted in many assays that the most clear-cut results are obtained with chicks up to 2 weeks of age. With older chicks, the syndrome develops more slowly and some may remain normal on deficient diets for comparatively long periods of time. Hence, we have adopted a 2-week test period, starting chicks on experimental diets as soon as hatched. This has a further advantage in conserving the basal diet and special supplement preparations.

To illustrate further the application of the preventive assay procedure, data on soybean oil as a vitamin K supplement are given in table 2.

In table 2 it is shown that 2% of soybean oil provided an adequate level of vitamin K, while 1% appeared slightly inadequate. This is the first report to our knowledge of the presence of vitamin K in soybean oil.

SUMMARY

1. A rapid procedure for assay of vitamin K supplements is described.

2. Determination of hemoglobin levels is unnecessary in such assay, since avitaminosis K is not a primary cause of anemia in chicks.

3. Vitamin K is present in soybean oil.

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SOURCES AND NATURE OF THE CHICK GIZZARD FACTOR

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Evidence for the existence of a fat-soluble factor required by the chick for the integrity of the gizzard lining has been presented in previous papers (Almquist and Stokstad, '36, '37). The existence of such a gizzard factor has also been the subject of reports by Kline, Bird, Elvehjem and Hart ('36) and Bird, Kline, Elvehjem, Hart and Halpin ('36). The latter workers have advanced the opinion that the factor is not fat-soluble, since attempts to extract it with ethyl alcohol and ethyl ether failed, and made the suggestion that the gizzard factor might prove to be identical with vitamin B_3.

It is the purpose of the present paper to provide further information on the distribution and properties of the chick gizzard factor.

METHODS AND RESULTS

The method followed was identical with that described in detail by Almquist and Stokstad ('37). The diet used was basal diet E supplemented with vitamin K in the form of alfalfa hexane extract treated with magnesium oxide to remove the gizzard factor. For simplicity, results have been condensed almost entirely to tabular form.

In table 1 are given the results of an experiment in which chicks were kept for 2 weeks on gizzard factor deficient diets and then for 4 weeks on supplemented diets.

The results of this experiment support the contention of Almquist and Stokstad ('36, '37) that the gizzard factor is fat-soluble. It remained in solution in hexane even after much fatty material had been removed by prolonged chilling at 0°C. followed by filtration at the same temperature. The solids removed were not active. The actual level of clarified wheat bran oil was 1.37% of the diet. This is the most active concentrate that has yet been obtained.

The result with dried normal gizzard lining is extremely interesting as it demonstrates the presence of the gizzard factor in the lining itself, and furnishes direct proof of the

TABLE 1

Average gizzard erosion score in chicks at 6 weeks of age

SUPPLEMENT	LEVEL	NUMBER OF CHICKS	AVERAGE GIZZARD EROSION, SCORE [1]	AVERAGE WEIGHT OF CHICKS
	%			*gm.*
None	.....	12	0.88	342.1
Wheat bran hexane extract, clarified by chilling	30 (equiv.)	12	0.21	338.2
Solids removed from wheat bran extract by chilling	30 (equiv.)	13	0.92	327.0
Dried gizzard lining from normal gizzards	5	12	0.08	355.0

[1] This score is identical with the average number of plus signs per gizzard used in a former paper (Almquist and Stokstad, '37).

existence of an anti-gizzard-erosion factor. The result has been confirmed by other experiments.

The growth results support the statement of Almquist and Stokstad ('37) that the gizzard factor is not a growth factor. There was no appreciable difference in the body weights attained by various groups although the gizzard erosions varied widely in severity between groups.

Further results on the distribution of the gizzard factor are given in table 2. In these experiments, chicks were placed on the various diets at 1 day of age and continued on these diets to an age of 4 weeks.

The results in table 2 offer further evidence that the gizzard factor is fat-soluble. The factor was found present in rice bran and in the hexane extract of rice bran but not in the hexane extracted rice bran. It was also found present in soybean oil which was a richer source than the soybean meal. Commercial soybean meals carry several per cent of the oil.

TABLE 2

Average gizzard erosion scores in chicks at 4 weeks of age

SUPPLEMENT	LEVEL	NUMBER CHICKS	AVERAGE GIZZARD EROSION, SCORE	AVERAGE WEIGHT OF CHICKS
	%			gm.
None	.....	8	1.12	220.0
Rice bran [1]	15	8	0.62	237.6
Rice bran [1]	25	11	0.45	214.5
Rice bran, hexane extracted [1]	15	8	0.94	218.6
Hexane extract of rice bran	30 (equiv.)	8	0.25	215.0
Kale, dried at 0°C.[1]	15	11	0.45	225.2
Kale, dried at 0°C.[1]	20	10	0.10	214.0
Barley, ground [1]	25	9	0.72	231.1
Alfalfa, dried [1]	20	11	0.32	146.4
Soybean meal [1]	15	10	0.40	225.0
None	.....	10	1.15	213.5
Peanut meal [1]	25	12	0.46	238.3
Sesame meal [1]	25	7	1.00	258.6
Hempseed meal [1]	25	10	0.05	247.5
Linseed meal [1]	25	6	1.17	81.7
Soybean meal [1]	25	11	0.23	....
Soybean oil no. 1 [1]	5	12	0.21	219.2
Soybean oil no. 1 [1]	10	8	0.20	211.0
Soybean oil no. 2 [1]	5	11	0.54	237.2
Soybean oil no. 2 [1]	10	8	0.19	216.3
Soybean oil no. 2, heated with ethyl alcohol [1]	10	10	0.85	204.2
Soybean oil no. 2, heated in vacuum for 24 hours at 120°C.[1]	10	10	0.90	223.0
Soybean oil no. 1, alcohol soluble fraction	10	8	1.38	217.0
Soybean oil no. 1, alcohol insoluble fraction [1]	10	8	0.86	225.0
Wheat bran [1]	25	10	0.45	256.0
Wheat bran, autoclaved at 120°C. for 24 hours [1]	25	10	1.10	230.0
Wheat bran, heated in vacuum at 120°C. for 24 hours [1]	25	9	1.33	221.0

[1] Used in replacement of an equal quantity of polished rice.

Hempseed meal had a strikingly unique effect on gizzard linings in addition to protecting them from erosions. These linings differed from all others that have been observed in that they were almost completely smooth and free from the ridges or folds usually found in a normal gizzard lining. Almquist and Stokstad ('37) have shown the presence of the gizzard factor in the hexane extract of hempseed. This extract, however, does not produce the peculiar smooth lining appearance noted with hempseed meal.

Evidence for destruction of the gizzard factor by alcohol is seen in the experiments with soybean oil heated to a refluxing temperature with ethyl alcohol, and with soybean oil that had been extracted several times with cold, 95% ethyl alcohol. Negative results were also obtained with the alcohol extract of soybean oil. These findings are in agreement with our previous experience in attempts to extract the gizzard factor. The use of ethyl and methyl alcohols, ethyl ether, or acetone has uniformly resulted in non-potent preparations indicating some destructive influence of these solvents.

The results with heated or autoclaved wheat bran and with heated soybean oil are in agreement with the report of Almquist and Stokstad ('36, '37) and Kline et al. ('36) that the factor is adversely affected by heating.

The average body weights of chicks given in tables 1 and 2 indicate definitely, in agreement with Almquist and Stokstad ('37), that the gizzard factor is not a growth factor. On the other hand, it has been stated by Kline et al. ('36) that absence of the gizzard factor leads to very poor growth, probably due to poor absorption. Further implications in regard to the effect of the gizzard factor on growth are to be found in the paper by Bird et al. ('36). Such a statement is difficult to understand in view of the fact that the gizzard is not an organ of absorption. Fritz, Burrows and Titus ('36) have shown with gizzardectomized fowls that the gizzard is not essential when fine ground feed is eaten.

Since the factor has been clearly demonstrated to be fat-soluble, in agreement with the first report from this laboratory, the supposition that the gizzard factor may be 'vitamin B_3' or any member of the vitamin B complex cannot be reconciled with the known properties of the factor. It seems possible, however, that more than one dietary factor may be concerned in the prevention of gizzard erosion.

SUMMARY

1. Additional information is presented on the distribution of the chick gizzard factor.

2. The gizzard factor is unstable to heat and to ethyl alcohol.

3. The gizzard factor is present in the gizzard lining.

4. The gizard factor has no noticeable relation to the rate of growth of chicks.

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VITAMIN A ACTIVITY OF BUTTERS DETERMINED BY VARIOUS METHODS[1]

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ONE FIGURE

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The vitamin A values of certain Kansas State College butters, determined by several methods developed in the past decade for quantitative estimation, are herein reported. Findings of 1) vitamin A and 2) carotene determinations were combined to estimate total vitamin A values and compared with 3) vitamin A values obtained by the single feeding method.

The work of Rosenheim and Drummond ('25) with $AsCl_3$ and Carr and Price ('26) with $SbCl_3$ led to numerous investigations of the resulting blue color as a test for vitamin A, often measured with a tintometer and also recently by means of the visual spectrophotometer (Morton, '35). Suitable preparation of the non-saponifiable portion (Baumann and Steenbock, '31, '33 b; Booth et al., '33) makes the Carr-Price test useful for butter.

Morton and Heilbron ('28) observed the presence of a specific absorption band at 328 mμ in oils, the intensity of the absorption being proportional to the vitamin A concentration.

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This method is suitable (Morton and Heilbron, '30; Baumann and Steenbock, '33 a) for study of the absorption maxima in butter.

Moore ('30) showed that the plant pigment carotene was converted into vitamin A in vivo. The determination of both vitamin A and its precursors is necessary for total vitamin A value in foods like butter which contain both. Carotene has been determined with the visual spectrophotometer (Baumann and Steenbock, '33 a) in melted butter fat or in butter dissolved in petroleum ether and also in the non-saponifiable portion dissolved in petroleum ether (Gillam and Heilbron, '35; Shrewsbury and Kraybill, '33), methods which permit the use of extinction coefficients (Skellysolve as solvent) based upon the international standard (Peterson et al., '37).

Biological assays, although time consuming, are considered valuable, to confirm results obtained in other ways, and fundamental, to give quantitative figures for the nutritive value of the vitamin and its precursor or precursors as used by the animal. The recently developed single feeding method (Sherman and Todhunter, '34), a modification of the widely used procedure of Sherman and Munsell ('25) has been found suitable for measuring the total vitamin A value of a product.

METHODS

The experimental butters were prepared in the Department of Dairy Husbandry, churned from sweet cream of 24-hour collections made on the first, fifth and thirtieth days of lactation and stored at 0°C. until used for experiment. The 'commercial sample' of butter was churned from milk delivered from neighboring farms and sold by the college dairy in the early part of February. Only this commercial sample contained added coloring matter, a vegetable compound which did not interfere with tests used, as shown by suitable checks.

Determination of vitamin A. 1. $SbCl_3$ spectrophotometric method. The unsaponifiable portion of each butter was prepared essentially as described by Morgan and associates ('35), using duplicate samples of 5 gm. of colostral butters or 15 gm.

of other butters. In preparing the solution for the spectrophotometer the proportion used by Gillam ('35) was maintained: 0.5 ml. of the unknown solution and 4.5 ml. of anhydrous $SbCl_3$ solution, prepared according to Wokes and Willimott ('27), were mixed and read against pure chloroform in less than 30 seconds at 583 mμ and 620 mμ, $E\,\{^{1\%\ \text{'pure' vitamin A}}_{1\ \text{cm.}}$ being equal to 2600 and 5000 (Morton, '35), respectively. Calculations were made by use of the Beer-Lambert law. Strain ('35) has stated that carotene gave a color reaction with $SbCl_3$, but with different maxima. This, however, was for concentrated solutions. That absorption here measured was due to vitamin A rather than to carotene was shown by running a control using an equivalent amount of carotene dissolved in Wesson oil in the above procedure in which case no color developed.

2. Spectrographic method. Duplicate samples were prepared (Baumann and Steenbock, '33 a; Semb, Baumann and Steenbock, '34). The vitamin A preparations, dissolved in methyl alcohol, sealed in an atmosphere of nitrogen, were kept in the dark until absorption spectra could be produced. The solutions were photographed with a Bausch and Lomb quartz spectrograph equipped with a Hilger rotating sector disc and a quartz biprism. The light source was a Ni-Fe arc, and the light was diffused through a frosted quartz plate. Values for $\log \frac{I_0}{I}$ at 328 mμ were obtained from duplicate plates and used in computing the value of $E\,\{^{1\%\ \text{butter}}_{1\ \text{cm.}}$, which were then compared with the accepted extinction coefficient for vitamin A, $E\,\{^{1\%\ \text{'pure' vitamin A}}_{1\ \text{cm.}}$, 328 mμ = 1600 (Carr and Jewell, '33) to calculate percentage of vitamin A in the butters (table 1). Corrections for the readings at 328 mμ have been suggested. De ('35) made spectrograms before and after exposing the sample to ultra-violet light for 3 hours and took the difference between the two absorptions to calculate vitamin A content. Morton ('35) described methods of correction and pointed out the interference of carotene and xanthophyll when chloroform is the solvent. However, as readings obtained when methyl alcohol is used as a solvent are usually accepted without correction, they are so presented here.

TABLE 1

Vitamin A and carotene determinations on butter, obtained by chemical and physical methods

BUTTER SAMPLES		VITAMIN A					β-CAROTENE			
		Spectrophotometric method, $SbCl_3$			Spectrographic method		Spectrophotometric method			
		620 mμ	583 mμ		328 mμ		480 mμ	470 mμ	455 mμ	
Source	Butter fat	1% 'pure' vit. A $E^{1\%}_{1\,cm.}$ = 5000	1% 'pure' vit. A $E^{1\%}_{1\,cm.}$ = 2600	Vitamin A	1% 'pure' vit. A $E^{1\%}_{1\,cm.}$ = 1600		1% β-carotene $E^{1\%}_{1\,cm.}$ = 2120	1% β-carotene $E^{1\%}_{1\,cm.}$ = 2000	1% β-carotene $E^{1\%}_{1\,cm.}$ = 2270	Carotene
	%	$E^{1\%\ butter}_{1\ cm.}$	$E^{1\%\ butter}_{1\ cm.}$	%	$E^{1\%\ butter}_{1\ cm.}$	vitamin A %	$E^{1\%\ butter}_{1\ cm.}$	$E^{1\%\ butter}_{1\ cm.}$	$E^{1\%\ butter}_{1\ cm.}$	%
Commercial	80.0	0.01560	0.01183	0.000383	0.01234	0.000771	0.00329	0.00334	0.00384	0.000164
Holstein 197										
Colostrum	89.5	0.07325	0.04591	0.001613	0.12010	0.007500	0.03788	0.03788	0.04222	0.001847
5th day	83.5	0.02200	0.01653	0.000506	0.01146	0.000710	0.00432	0.00444	0.00520	0.000218
30th day	84.0	0.01280	0.00775	0.000277	0.00919	0.000568	0.00583	0.00570	0.00663	0.000284
Jersey 342										
Colostrum	79.5	0.04115	0.02501	0.000892	0.08010	0.005000	0.05975	0.05950	0.06900	0.002945
5th day	90.0	0.02380	0.01510	0.000528	0.01975	0.001228	0.02251	0.02238	0.02624	0.001112
30th day					0.00644	0.000396	0.00225	0.00238	0.00329	0.000123
Jersey 378										
Colostrum	84.5	0.05900	0.04030	0.001365	0.06885	0.004297	0.04566	0.04534	0.05384	0.002264
5th day	91.0	0.01805	0.01258	0.000423	0.00978	0.000605	0.01024	0.01062	0.01226	0.000518
30th day	86.0	0.00785	0.00525	0.000179	0.00719	0.000443	0.00738	0.00750	0.00824	0.000362

Determination of carotene. Portions of 5 or 10 gm. of butter were dissolved directly in 25 ml. of petroleum-ether (Shrewsbury and Kraybill, '33). No correction was necessary for the butter fat dissolved in the Skellysolve, as determined by readings on samples decolorized with norite. The solution was filtered, made up to 100 ml., and read in a visual spectrophotometer at 455, 470 and 480 mμ. The extinction coefficients, $E\{^{1\%\ \beta\text{-carotene}}_{1\ \text{cm.}} = 2270$, 2000 and 2120, respectively, determined by Peterson et al. ('37) for β-carotene in Skellysolve, were used. Gillam and Heilbron ('35) suggested that the xanthophyll be separated from the carotene by partition between 90% methyl alcohol and light petroleum ether. Gillam ('34) found the proportion of xanthophyll to carotene to be fairly constant in butter fat and to represent a ratio of 1:14 by weight, or approximately 6% of the total yellow coloring matter. In later routine work he did not perform the separation but simply corrected for the xanthophyll. Other authors feel that it is unnecessary to correct for xanthophyll, and carotene figures in this study are presented uncorrected.

Determination of total vitamin A value. The single feeding method of Sherman and Todhunter ('34) was employed for biological assay. Young rats of Wistar Institute strain, weighing 38 to 40 gm., were depleted of vitamin A reserves (average depletion period = 41 days) and divided into experimental groups of ten or more animals. Carotene (international standard for vitamin A held for the Health Section of the League of Nations by the National Institute for Medical Research, Hampstead, London, N.W. 3) secured through the Bureau of Chemistry and Soils, United States Department of Agriculture, was dissolved in cottonseed oil (Wesson oil, Baumann and Steenbock, '33 b) and stored in the dark below 0°C. until needed. Portions of this solution equivalent to 34 γ, 17 γ and 10 γ pure β-carotene were given as single feedings. Composite growth curves (fig. 1) were plotted so that areas could be measured in the usual way with the planimeter and comparisons made with the carotene reference curves.

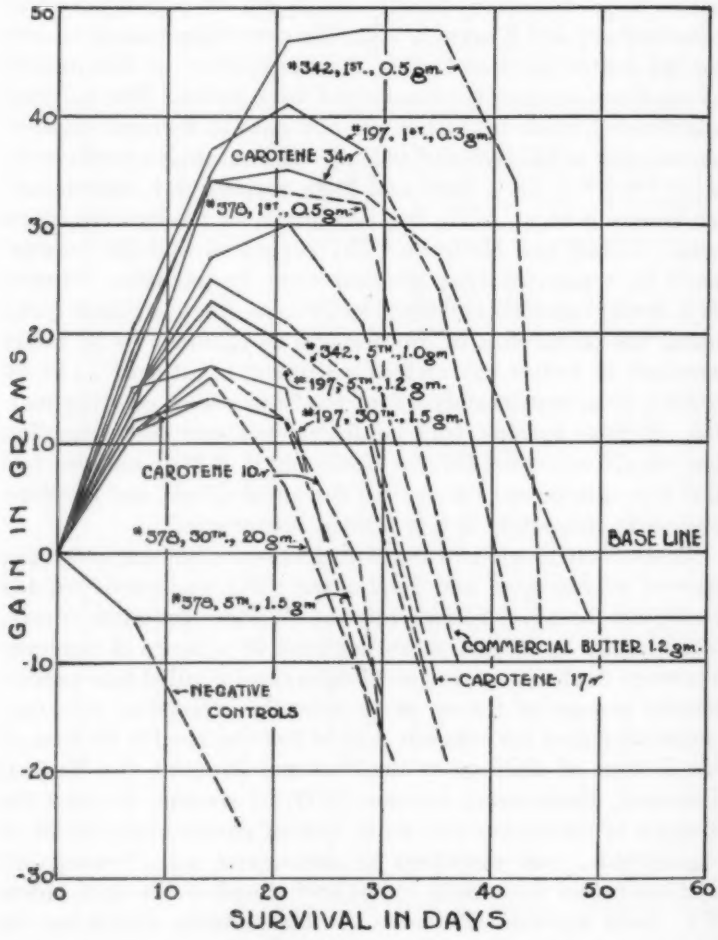

Fig. 1 Average weight curves of rats, previously depleted of body store of vitamin A, to which single feedings of butter and carotene have been given, as indicated.

DISCUSSION

Results with $SbCl_3$ gave information (table 1) regarding the relative richness in vitamin A of the various samples and definitely distinguished the butters of high from those of low vitamin A content. In agreement with the work of Morgan et al. ('35), who have discussed the limitations of the $SbCl_3$ method, results obtained from the 583 mμ readings were in every case higher than those from 620 mμ. According to Morgan et al. ('35) vitamin A values obtained with $SbCl_3$ tend to represent lower limits on account of inhibition while values obtained at 328 mμ indicate upper limits. Our findings are in accord with this for every butter tested, the values at 328 mμ being invariably distinctly higher than those obtained with $SbCl_3$.

The butters produced on the first day of lactation were richest in β-carotene. The commercial butter contained more β-carotene than that produced by cow 342 on the thirtieth day of lactation but less than the thirtieth day butters of the other two cows.

Table 2 summarizes 1) data obtained by biological assay, used to estimate the total vitamin A value of the butters, and 2) data from feeding the international standard β-carotene used for the carotene determinations. The areas under the composite curves have been shown by the originators of the method to be directly proportional to the vitamin value of the single feeding. The results of feeding three levels of carotene indicated on the table, show that this expected ratio of areas holds for the animals of the present series, confirming the validity of the method as used in our laboratory.

A summary of the findings obtained from the preceding tables expressed in gamma and as international units (Nelson, '35) is presented in table 3. Total vitamin A value is recorded in three ways: 1) the summation of the results from the $SbCl_3$ tests and from the carotene determinations, 2) the summation of results obtained from the intensity of absorption at 328 mμ and from the carotene determinations and 3) the biological assay findings. The three sets of figures show interesting

TABLE 2

Total vitamin A value determined by biological assay

SAMPLE	RATS USED		AMOUNT FED	AVERAGE LENGTH OF SURVIVAL	AREA	RESULTS PER GRAM OF BUTTER FED		VITAMIN A VALUE OF BUTTER
	Number	Average weight when depleted				Area	Value in β-carotene	
		gm.	*gm.*	*days*	*sq.in.*	*sq.in.*	γ	%
Commercial	9	94	1.2	37	8.25	6.88	16.99	0.001812
Holstein 197								
Colostrum	13	92	0.3	41	13.40	44.67	110.33	0.011769
5th day	8	95	1.2	36	5.75	4.79	11.83	0.001262
30th day	10	93	1.5	29	4.19	2.80	6.92	0.000738
Jersey 342								
Colostrum	11	92	0.5	44	17.77	35.54	87.78	0.009363
5th day	11	91	1.0	34	6.41	6.41	15.83	0.001689
Jersey 378								
Colostrum	13	93	0.5	37	10.00	20.00	49.40	0.005369
5th day	10	94	1.5	30	4.36	2.91	7.19	0.000767
30th day	10	94	2.0	31	3.45	1.73	4.27	0.000455
Carotene	11	91	10 γ	30	4.14	1 sq.in. = 2.41 γ pure β-carotene		
Carotene	23	92	17 γ	35	6.73	1 sq.in. = 2.48 γ pure β-carotene		
Carotene	9	92	34 γ	47	13.69	1 sq.in. = 2.53 γ pure β-carotene		
Negatives	17	93		17		Av. = 2.47 γ		

TABLE 3

Summary of total vitamin A values per gram of butter fat and per 24-hour yield obtained by chemical, physical and biological methods

SAMPLE	VALUES OBTAINED BY								
	$SbCl_3$ + carotene determinations			328 mμ + carotene determinations			Biological assay		
	Per gram butter fat		Per 24-hour yield	Per gram butter fat		Per 24-hour yield	Per gram butter fat		Per 24-hour yield
	γ	I.U.	I.U. (1000 S)	γ	I.U.	I.U. (1000 S)	γ	I.U.	I.U. (1000 S)
Commercial	6.98	11		11.83	18		22.65	35	
Holstein 197									
Colostrum	41.86	65	28	105.81	165	72	131.50	205	89
5th day	8.85	14	15	11.29	18	20	15.11	24	26
30th day	6.91	11	14	10.37	16	20	8.79	14	18
Jersey 342									
Colostrum	50.73	79	31	102.40	160	63	117.77	184	73
5th day	19.04	30	15	26.82	42	21	18.77	29	14
Jersey 378									
Colostrum	43.55	68	8	78.25	122	15	63.54	99	12
5th day	10.73	17	12	12.73	20	14	8.43	13	9
30th day	6.57	10	9	9.64	15	13	5.29	8	7

correspondence for results obtained in diverse ways. The second summation most often includes the highest figures for total vitamin A value whereas the first summation often includes the lowest of the three figures for any one sample. By any of the three methods, the colostral butters are shown to contain butter fat of highest vitamin A value, which is consistent with the findings of other workers (Dann, '33; Semb et al., '34; Gillam et al., '36). In the colostral butters studied, 20 to 40% of the total vitamin A value appeared to be due to the carotene and in some later butters a good proportion of the vitamin A value was supplied by precursors. The commercial butter had one of the lower carotene contents, accounting for about 19% of the total vitamin A value. This butter was churned from milk delivered at the dairy from neighboring farms on which the rations fed may have been deficient in good quality roughage as a result of the drought, whereas the other samples were made from milk of cows on well-balanced rations including liberal amounts of alfalfa hay and silage. Baumann and Steenbock ('33 a) found that the carotene in a series of Wisconsin butters, prepared throughout the year, accounted for an average of 15% of the vitamin A value, and later Baumann et al. ('34) reported large proportions of the vitamin A value to be due to carotene, about half for some Jersey samples and less for Holstein samples. Gillam et al. ('36) investigated the carotene and vitamin A contents of the colostrum of six cows kept under typical English conditions and reported well over half the vitamin A value due to carotene in five instances.

Data from dairy records were used to compile yields (table 3). Cow 378 was slow in starting her lactation and her yield was considerably lower than the other cows which accounts for the apparently low total production of vitamin A on the first day of lactation. Other cows furnishing samples for this experiment produced between 60,000 and 90,000 I.U. of vitamin A value on the first day of lactation. As lactation progressed the total daily yield of butter fat increased but the vitamin A value of the butter fat dropped so that the total

output of vitamin A value on the thirtieth day of lactation was distinctly lower. Dann ('33) found extreme differences between the vitamin A values of colostrum and standard milk, determined by colorimetric methods, and concluded that a calf receives on the first day of life supplies of vitamin A greater than later milk could give in 20 to 50 days. The present investigation of butter shows that the total vitamin A value of the colostrum of the first day of lactation may be four or five times as great as that of milk of the thirtieth day for the same cow.

SUMMARY AND CONCLUSIONS

Vitamin A values were determined for a 'commercial' winter butter and for butters made from composite 24-hour samples of the secretions of the first, fifth and thirtieth days of lactation of three individual cows, a Holstein and two Jerseys. The vitamin A content was estimated by measuring intensity of the blue color, obtained with the $SbCl_3$ reagent added to the unsaponifiable fraction, in a visual spectrophotometer and also the absorption spectra at 328 mμ with a quartz spectrograph (courtesy physics department, Iowa State College). The amount of carotene was measured spectrophotometrically with petroleum ether (Skellysolve) as the solvent. The vitamin A value, or combined activity of vitamin A and precursors, was determined by the single feeding method of biological assay, giving results expressed as international units of vitamin A, by reference to curves of animals fed standard β-carotene.

The $SbCl_3$ tests yielded information regarding the relative amounts of vitamin A in the various samples, distinguishing the butters of high from those of low vitamin A content. These findings with $SbCl_3$ on butter tended to represent definitely lower limits of vitamin A content whereas the results at 328 mμ represented the upper limits. The more satisfactory measurements at 328 mμ showed that the butter fats produced on the fifth day of lactation contained from one-fourth to one-tenth the amount of vitamin A per gram found in that produced by the same cow on the first day. Butter fats

produced on the thirtieth day of lactation showed further decreases in vitamin A content. The sample of commercial butter had a vitamin A content similar to those of later butters.

Readings with the visual spectrophotometer showed that the carotene contents of the colostral butters were definitely superior to those of the other butters. The carotene contents dropped during the course of lactation, as indicated by the tables. The carotene content of the commercial butter was similar to those of thirtieth day samples studied.

Biological assay showed the butter fat of colostrum to be richest in total vitamin A value for each cow studied. Butter fats produced on the fifth day of lactation were only one-sixth to one-ninth as potent while those produced on the thirtieth day of lactation were even lower in vitamin A value, as indicated.

Total vitamin A values per gram of butter fat, determined by the single feeding method, were compared to the summations of values obtained by chemical and physical methods, namely, $SbCl_3$ plus carotene and 328 mμ plus carotene. In some cases the summations including vitamin A determined with $SbCl_3$ were similar to the biological results while in other instances these summations were distinctly lower which is consistent with the view that the $SbCl_3$ readings represent lower limits for vitamin A content. The summations including the determinations of vitamin A at 328 mμ plus carotene values were on the whole in better agreement with the results of biological determinations of total vitamin A activity. Results of determinations at 328 mμ plus β-carotene values compare well with results of biological determinations by the single feeding method, and apparently account for most of the vitamin A value of the butter fats examined.

Results from these methods show that two samples of butter fat from colostrum contained about 175 international units of vitamin A value per gram and the other more than 100 units. Samples produced by the same cows on the fifth day of lactation were found to contain only one-fifth to one-ninth this amount. By any of the three methods of measurement, butter

fat of the thirtieth day of lactation was lower in vitamin A value than that of the fifth day of lactation. Total daily yields of vitamin A value were calculated for the individual cows; two of these produced between 60,000 and 90,000 international units on the first day of lactation while the third had a lower production. As lactation progressed the vitamin A value of the daily yield of butter fat dropped, that of the fifth day being lower than that of the first and the output on the thirtieth day showing some further decrease.

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THE INFLUENCE OF SOME COMMONLY USED SALT MIXTURES UPON GROWTH AND BONE DEVELOPMENT OF THE ALBINO RAT [1]

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A suitable mixture of inorganic salts forms a necessary part of any experimental diet composed of purified foodstuffs if this diet is to be adequate for normal nutrition. Early reports of attempts to prepare rations consisting wholly of purified foodstuffs indicated this need. The various combinations of salts employed by such workers as Lunin (1881), Socin (1891), and Hall (1896), were in large measure suggested by the analyses of milk by Bunge (1874). These investigators, as well as others, tried to construct mixtures that would provide the essential inorganic constituents of milk, and a wide variety of chemical compounds appears to have been used. A typical mixture is that recorded by Socin:

	gm.
Potassium carbonate	12.5
Calcium phosphate	20.3
Calcium carbonate	7.1
Magnesium chloride	2.7

Another early one, that of Munk (1893) shown below, was modelled after the 'Fleischasche' analyses of Wolff (1871,

[1] A preliminary report was presented before the American Institute of Nutrition at the Detroit meeting, April, 1935.

The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

1880), and is, as a result, quite different from those based on Bunge's milk analyses.

	gm.
Potassium phosphate	70
Sodium chloride	15
Calcium phosphate	6
Magnesium phosphate	8
Iron oxide	0.5

The investigators who used these mixtures were interested in a variety of metabolism experiments which need not be reviewed here. It is quite evident from the reports, however, that the partial failure of many studies was attributed to inadequacies in the salt mixture used, and some of the later workers attempted to correct the deficiencies by adding the ash of some animal tissue to the various combinations of inorganic salts. For example, Falta and Noeggerath ('06) added milk salts and the salts of horse serum to the mixture described by Socin.

In one of the early publications from this laboratory (Osborne and Mendel, '11), three salt mixtures are mentioned. The first was essentially that introduced by Röhmann in 1902 with the addition of ferric citrate, and differed from some of the others in that it included several organic salts. The second was suggested by the work of McCollum ('09) who supplemented the purified protein, fat, and carbohydrate of his experimental diet with 5% ash of milk, 2% calcium phosphate, and 1% sodium chloride with the addition of ferric chloride 'at intervals.' The third was a modification of that described by Henriques and Hansen ('05) with ferric citrate as an additional component.

The composition of these mixtures as used by Osborne and Mendel is shown in table 1.

In 1911, Osborne and Mendel began their studies of the relative nutritive value of proteins as determined by growth. As they reported at that time, earlier trials with purified protein, fat, carbohydrate and modified Röhmann salts had been successful only in maintaining the weight of experimental animals. On the other hand, growth had resulted when they fed a paste of milk powder, lard and starch. They accordingly turned their attention to the non-protein constituents of

milk. The product designated as 'protein-free milk' was prepared and, although it contained lactose in addition to the milk salts, was administered with the intention of supplying the needs of the animals for inorganic salts.

The success that followed the substitution of 'protein-free milk' for the salt mixture that had previously been used was so marked that Osborne and Mendel decided to investigate this particular fraction further in an attempt to explain the improvement. As they pointed out in 1912, it seemed possible that the difference might be due to the fact that the mixture of pure salts did not "represent precisely the composition of the milk salts as they occur in their natural medium," and

TABLE 1

Salt mixtures used by Osborne and Mendel in early investigations

SALT MIXTURE I		SALT MIXTURE II		SALT MIXTURE III	
	gm.		*gm.*		*gm.*
$Ca_3(PO_4)_2$	10.0	Ash of milk	60.6	NaCl	33.4
K_2HPO_4	37.0	$Ca_3(PO_4)_2$	24.2	KCl	33.4
NaCl	20.0	NaCl	12.1	Bone ash	25.1
Na citrate	15.0	Fe citrate [1]	3.1	Na_2CO_3	6.7
Mg citrate	8.0		100.0	Fe citrate [1]	1.4
Ca lactate	8.0				100.0
Fe citrate [1]	2.0				
	100.0				

[1] Fe citrate added by Osborne and Mendel.

that "a mixture of crystalline salts containing the various ions present in the protein-free milk might present an opportunity for an unequal rate of absorption of the inorganic salts from the ingested food." Consequently, they prepared 'artificial protein-free milk' (Osborne and Mendel, '12) by dissolving inorganic salts in a water solution of hydrochloric, phosphoric, citric, and sulfuric acids; lactose was added and the mixture was evaporated and dried. When this product was used to replace 'protein-free milk' in the food, growth was superior to that obtained with salt mixture I in the diet, but was not as satisfactory as that observed when natural 'protein-free milk' was used. The explanation of the superiority of the diets that contained 'protein-free milk' over those

containing the artificial product has been discussed elsewhere (Osborne and Mendel, '16, '17 a, '17 b). The significant fact in connection with the present paper is that 'artificial protein-free milk,' prepared without the addition of lactose, has been known for many years as Osborne and Mendel salts ('17 c, '19).

This salt mixture, as well as many others that have been described, has been widely used to supply the inorganic constituents in rations composed of purified foodstuffs. In some laboratories it has been found inconvenient to prepare the Osborne and Mendel salt mixture as they described it and, as a result, two modifications (Hawk and Oser, '31; Wesson, '32) have been suggested, both prepared by mixing appropriate dry salts. In each case the relative proportions of the elements are substantially the same as in the original.

The essential differences in elementary composition between these and some of the other well-known salt mixtures (McCollum and Davis, '14; Steenbock and Nelson, '23; Sure, '24) are shown in table 2. The figures represent the grams of each element in 4 gm. of salts, the amount usually included in 100 gm. of food. It is evident that these mixtures vary in the number and proportions of the elements included. Both the McCollum and Sure salts are made without iodine, but this is supplied in the drinking water. Of the four, only that of Osborne and Mendel contains added trace elements other than iodine, and none of them contains added copper.

These salt mixtures, particularly the Osborne and Mendel and the McCollum salts, are frequently used interchangeably, as in the United States Pharmacopoeia XI method for assaying vitamin A in cod liver oils. Such variations in the composition of a food as are produced when one of these mixtures is substituted for another, may not represent serious uncontrolled variables in some types of experiments. However, in any work involving the metabolism of inorganic salts, it is important to ascertain whether such differences as are indicated in table 2 may or may not be disregarded. This is especially necessary when dealing with the greatly enhanced rates of growth that have been observed in albino rats in

recent years (Osborne and Mendel, '26; Mendel and Hubbell, '35).

With regard to the change in rate of growth of this laboratory animal, it is interesting to note that Osborne and Mendel stated in 1911, "within one month a 70-gram white rat ordinarily will double its weight when the diet is adequate." So small a rate of gain is regarded as decidedly subnormal in

TABLE 2

Calculated composition of four common salt mixtures

	OSBORNE-MENDEL [1] (17)	McCOLLUM [2] (20)	STEENBOCK (21)	SURE [3] (22)
	gm.	*gm.*	*gm.*	*gm.*
Calcium	0.462	0.276	0.296	0.322
Magnesium	0.0597	0.0581	0.0398	0.0681
Sodium	0.128	0.136	0.226	0.159
Potassium	0.684	0.463	0.513	0.541
Phosphorus	0.279	0.412	0.458	0.482
Chlorine	0.444	0.114	0.233	0.132
Sulfur	0.0259	0.0766	0.0525	0.0897
Iron	0.0112	0.0263	0.0101	0.0309
Iodine	0.000131		0.0020	
Manganese	0.000246			
Fluorine	0.000961			
Aluminum	0.0000219			

Data are in grams contained in 4 gm. of the mixture. the quantity usually included in 100 gm. of complete diet.

[1] Due to moisture these figures are lower than the theoretical values. They represent the composition of the salt mixture as used in these experiments.

[2] Iodine supplied in drinking water.

[3] First described by Steenbock as no. 32 salts. Iodine was supplied in the drinking water by Sure.

our colony today, and it seemed desirable to investigate the dietary requirements, in order to determine whether rations formerly considered adequate are suitable for an animal that gains 5 gm. a day instead of 2. The first step has been an examination of the inorganic constitutents of the diet, to ascertain to what extent some of the common salt mixtures fulfill the requirements for this more rapid development.

With this in view, the four salt mixtures described in table 2 were selected for detailed study. Inasmuch as a mixture may appear satisfactory when fed in an amount that assures liberal intake of the various ingredients, but may give rise to nutritive disturbances when the proportion in the diet is markedly increased or decreased, the four mixtures have been compared at various levels of intake. By decreasing the amount fed from the usual 4%, any lack or disproportion in the elements may become more evident. On the other hand, harmful effects due to an excess of any of the constituents may become sufficiently obvious to be noted if the amount fed is increased considerably above that ordinarily used. Consequently all four mixtures have been supplied at the following levels: 0.5, 1, 2, 3, 4, 5, 10 and 15% of the ration.

The bones are the largest storehouse for minerals in the body, and may be expected to reflect changes in the salt intake, particularly with respect to calcium and phosphorus. We have chosen, therefore, to evaluate the efficiency of the various mixtures not only by the rate of change in body weight during a selected period of active growth (60 to 200 gm.) but also by the composition of the bones. Preliminary experiments had indicated that the ash of the dry fat-free femurs should approximate 58 to 60% in the case of rats that make a daily gain of 5 gm. or more during the period specified.

The experimental procedure was the same for all groups studied. Male rats were weaned at 21 days of age, at an average body weight of 45 gm., and were placed in groups of ten on diets that contained casein 18, butter fat 9, lard 20, and dried yeast 8%. The remaining 45% of the ration consisted of starch and the salt mixture to be investigated; 10 drops of cod liver oil were supplied as a daily supplement, and distilled water was always available. Records were kept of food consumption, but no attempt was made to control the amount eaten. All animals were killed by ether at 200 gm. body weight and the paired femora were analyzed.

METHODS OF ANALYSIS

The femora from each animal were freed from tissue and dried at 105 to 107° for 4 days. The dry bones were then cracked in a mortar, transferred to a weighed ignited Gooch crucible and extracted for 2 days with 95% alcohol, and then for 2 days with ether. The extracted bones were dried at 105 to 107° for 6 to 8 hours and the weight of the 'fats' was obtained. The fat-free bones were then charred over a low flame and heated overnight in an electric muffle at about 600°, and the percentage of ash in the dry fat-free bones was calculated.

DISCUSSION

The data are summarized in table 3. In making comparisons of one salt mixture with another, it has been necessary to consider variations in the rate of growth within any one group. It has not been possible in this paper to include data for individual animals, but the ranges, both for the rate of gain per day and for the ash in the dry fat-free bones are given. Of the ten rats that received a diet containing 3% Osborne and Mendel salt mixture, one grew at a rate of 4.5 gm. a day and another at the rate of 6.6 gm. a day. The bone ash for the former was 59.9% in contrast to 56.1% for the latter. This difference in the mineral matter of the bones was accompanied by a corresponding difference in food intake. The more slowly growing rat consumed 350 gm. of food while the other ate but 278 gm. Similar differences in food intake help to explain the variations in bone ash in other groups.

At the level of salts most commonly used, 4% of the ration, the average rate of growth was the same for the Osborne and Mendel and for the McCollum salts, namely 5.2 gm. a day for the gain from 60 to 200 gm. body weight. The percentage of ash in the dry, fat-free bones was somewhat higher in the former group, however, being 60.8% in contrast to 57.9%. Both values, however, were of the desired magnitude for this rate of growth. For the animals on the Steenbock and the Sure salts, the bone ash figures were somewhat higher than for those fed the McCollum salts. This was accompanied by a

TABLE 3

Comparison of the effect of salt mixtures supplied at different levels in the diet[1]

SALT MIXTURE IN DIET	DAILY GAIN		TOTAL FOOD INTAKE, AVERAGE	TOTAL Ca INTAKE,[2] AVERAGE	TOTAL P INTAKE,[2] AVERAGE	ASH IN DRY FAT-FREE FEMURS	
	Range	Average				Range	Average
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	%	%
1%							
Osborne-Mendel	2.9–4.5	3.6	372	0.51	1.17	44.4–48.8	46.5
McCollum	2.3–4.0	2.8	439	0.40	1.52	42.9–49.1	46.2
Steenbock	2.4–4.0	3.0	436	0.41	1.57	42.2–49.4	46.2
Sure	2.5–4.7	3.0	454	0.45	1.66	42.1–51.6	46.5
2%							
Osborne-Mendel	5.2–6.4	5.8	298	0.75	1.15	49.7–53.2	51.8
McCollum	3.9–5.8	4.5	336	0.54	1.51	48.2–51.6	50.1
Steenbock	3.2–7.0	4.0	363	0.61	1.72	45.9–53.4	50.1
Sure	4.0–6.1	5.0	316	0.57	1.53	48.6–51.2	50.0
3%							
Osborne-Mendel	4.5–6.6	5.4	308	1.13	1.40	56.1–59.9	57.5
McCollum	4.2–6.1	4.8	330	0.76	1.82	52.8–57.6	55.5
Steenbock	3.4–6.4	4.5	325	0.79	1.91	52.9–57.2	55.4
Sure	3.5–7.0	5.0	304	0.79	1.84	50.9–59.3	54.4
4%							
Osborne-Mendel	4.2–6.4	5.2	324	1.57	1.70	59.5–62.4	60.8
McCollum	3.8–7.7	5.2	309	0.93	2.02	56.3–60.0	57.9
Steenbock	4.0–5.2	4.7	329	1.04	2.31	57.9–60.5	59.2
Sure	2.8–6.4	5.0	306	1.04	2.22	57.4–60.9	59.0
5%							
Osborne-Mendel	4.5–6.6	5.4	309	1.85	1.84	57.5–61.0	59.9
McCollum	4.4–5.6	4.8	323	1.20	2.44	58.1–61.6	60.3
Steenbock	4.4–6.4	5.4	295	1.15	2.41	57.1–60.6	58.7
Sure	3.8–5.8	4.7	337	1.42	2.85	57.2–61.8	59.9
10%							
Osborne-Mendel	4.7–6.1	5.6	308	3.63	2.91	58.8–62.2	61.1
McCollum	2.9–5.4	4.2	340[3]	2.45[3]	4.30[3]	59.6–62.8	61.3
Steenbock	3.4–5.2	4.1	356[3]	2.71[3]	4.95[3]	60.1–62.5	61.4
Sure	2.3–5.2	3.4	441	3.62	6.37	60.1–64.6	62.5
15%							
Osborne-Mendel	2.7–4.8	4.0	361[3]	6.35[3]	4.68[3]	60.7–65.0	63.0
McCollum	2.1–5.2	3.2	454[3]	4.86[3]	8.06[3]	59.6–65.0	62.4
Sure	2.8–3.2	3.0	413[3]	5.04[3]	8.45[3]	62.5–63.7	63.2

[1] Ten rats were used in each group except that with 15% Sure salts. There were five in this group.

[2] In all cases 100 gm. of food without the addition of salts supplied 0.020 gm. Ca and 0.245 gm. P.

[3] Food spilled and figures are estimates.

slightly lower growth rate in both cases. As the amount of salt mixture in the ration was decreased to 2 or 3%, the differences were still apparent as long as the rate of growth was maintained at approximately 5 gm. a day. In all cases, there appeared to be more complete calcification with the salt mixture that contained the higher proportion of calcium. When the salt intake was reduced to 1% of the ration, growth was so slow, an average of 3.1 gm. a day for all mixtures, that the differences in degree of calcification disappeared. The results obtained when each salt mixture was used as 0.5% of the diet have not been included in the summary because so few animals reached the required weight of 200 gm. It is perhaps significant, however, that eight out of a possible ten in the group which received 0.5% Osborne and Mendel salt mixture did reach 200 gm. Growth was slow, being but 2.6 gm. a day, and the bone ash was 40.9%, which is below the average figure for 21-day-old rats (42.8%). In addition, there were many signs of disturbed nutrition, such as diarrhoea, scattering of food, loss of hair, distorted bones, humped backs, etc. With the other salt mixtures, these disturbances were much more marked and there were also many cases of bloody eyes, sore legs, legs partly paralyzed, and general irritability. Several of the animals died before they reached 100 gm. in weight, and most of the others were killed when it became obvious that it would take so long for them to reach 200 gm. that comparisons with more rapidly growing rats would not be significant. When the McCollum salt mixture was used at this level (0.5%), one rat reached 200 gm. in 57 days, or at the rate of 2.4 gm. a day and the bone ash was 34.0%. Four animals fed the diet that contained 0.5% Sure salts completed their required gain in 73 days (1.9 gm. a day), and the bone ash was 43.0%. Although they grew at that greatly reduced rate, the animals were just able to maintain the amount of ash present in the bones at weaning.

When the amount of inorganic salts included in the ration was raised above the usual 4% level, it was evident that there was enough of all constituents present to insure adequate

calcification with all salt mixtures, independently of the rate of growth.

Attention should be called to the fact that, at the levels of 2 and 3%, growth compares quite favorably with that obtained when the larger amounts were used. It is thus evident that a lowering of the total amount of salts has no harmful effect on growth rate, but at these lower levels there is in no case sufficient calcium to ensure adequate bone formation for the rate of growth maintained.

The fact that satisfactory growth could be obtained with a total intake of 3 or even with 2% of salts, suggested that it

TABLE 4

Effect of increasing the calcium content of salt mixtures [1]

SALT MIXTURE IN DIET [2]	TOTAL Ca IN SALTS	AVERAGE DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE [3]	TOTAL P INTAKE [3]	ASH IN DRY FAT-FREE FEMURS
	gm.	*gm.*	*gm.*	*gm.*	*gm.*	%
Osborne-Mendel + $CaCO_3$	0.58	5.8	288	1.73	0.91	59.5
McCollum + $CaCO_3$	0.58	5.8	318	1.91	1.11	58.5
Steenbock + $CaCO_3$	0.58	5.6	315	1.89	1.13	57.7
Sure + $CaCO_3$	0.58	5.0	334	2.00	1.22	58.9

[1] Ten rats were used in each group.

[2] In each case 1 gm. of the salt mixture was supplemented with sufficient calcium carbonate to furnish an amount of calcium equal to that in 5 gm. of Osborne and Mendel salts.

[3] In all cases 100 gm. of food without the addition of salts supplied 0.020 gm. Ca and 0.245 gm. P.

might be possible to prepare a salt mixture that would give the desired calcification when used in these smaller proportions. Consequently, to study the limiting factor or factors which prevent adequate calcification when the salt mixtures are used at low levels, a series of experiments was conducted in which various supplements were added to the original preparations. Only one of these is included inasmuch as it has the most direct bearing on the composition of our new salt mixture. To 1% of each of the four mixtures, calcium carbonate was added in sufficient quantity to furnish an amount of calcium equal to that supplied when 5% of Osborne and Mendel salts

is included in the diet. With this additional calcium, growth was rapid (5.0 to 5.8 gm. daily), and calcification (57.4 to 58.9%) was comparable to that produced when the unsupplemented salt mixtures were supplied as 4% of the diet. Table 4 shows the close agreement obtained in all cases.

SUMMARY AND CONCLUSIONS

Four common salt mixtures, the Osborne and Mendel, McCollum's 185, Steenbock's 40, and Sure's modification of the Steenbock 32 salts have been compared. As judged by rate of growth from 60 to 200 gm., and percentage of ash in the dry fat-free bones, the four mixtures are not interchangeable at any of eight levels studied. In spite of more rapid growth, higher bone ash was in general attained with the Osborne and Mendel salt mixture. Evidence is presented to show that, if adequate calcium is supplied, many other constituents can probably be furnished in amounts considerably below those ordinarily used.

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A NEW SALT MIXTURE FOR USE IN EXPERIMENTAL DIETS [1]

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A comparison of four salt mixtures in common use, namely the Osborne and Mendel, McCollum's 185, Steenbock's 40, and a modification of the Steenbock 32 salts, was reported in the preceding paper ('37). As judged by the criteria used, daily gain in weight during a selected period of active growth, and percentage of ash in the dry, fat-free femurs, the mixtures are not interchangeable in the diet of the albino rat at the various levels studied. It was also demonstrated that with each salt mixture, greater uniformity in respect to rate of growth and proportion of bone ash could be attained if the relative amount of calcium was considerably increased. When this was done, the total salt intake could be reduced from 4 to 2%. The bone ash produced under these conditions was sufficiently high to indicate that it should be possible to construct a mixture that could be successfully used at this lower level. The experimental work on which such a salt mixture is based is described in the present paper. Inasmuch as the new preparation is designed for use as a substitute for the Osborne and Mendel salts, a comparison of these two mixtures has been made under various conditions.

[1] The expenses of this investigation were shared by the Connecticut Agricultural Experiment Station and the Carnegie Institution of Washington.

[2] Part of the experimental work reported in this paper was completed after the death of Professor Mendel. Prof. Arthur H. Smith has generously advised us during much of the investigation, and it is with pleasure that we acknowledge his cooperation.

The general plan of supplementation described in the preceding paper was employed to determine a suitable amount of Osborne and Mendel salts to be used as a basis for the modified mixture. Three amounts of the salt mixture, 0.5, 1 and 2 gm. per 100 gm. of food, were supplemented with calcium carbonate in an attempt to ascertain the quantities of the other ions required when the relative proportion of calcium is markedly increased. In the case of 0.5% of Osborne and Mendel salts, enough calcium carbonate was added to

TABLE 1

Effect of adding calcium to various levels of Osborne and Mendel salt mixture[1]

SALT MIXTURE IN DIET	TOTAL Ca IN SALTS	DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE[2]	TOTAL P INTAKE[2]	ASH IN DRY FAT-FREE FEMURS
%	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%
0.5	0.06	2.2	518[3]	0.41[3]	1.45[3]	40.3
0.5 + $CaCO_3$	0.23	3.8	360	0.90	1.01	52.1
2	0.23	5.8	298	0.75	1.15	51.8
1	0.12	3.6	372	0.51	1.17	46.5
1 + $CaCO_3$	0.46	5.2	330	1.60	1.04	59.2
2 + $CaCO_3$	0.46	5.2	301	1.46	1.16	58.5
4	0.46	5.2	324	1.57	1.70	60.8
1 + $CaCO_3$	0.58	5.8	288	1.73	0.91	59.5
2 + $CaCO_3$	0.58	4.7	333	2.00	1.28	61.0
5	0.58	5.4	309	1.85	1.84	59.9

[1] Ten rats were used in each group.

[2] In all cases 100 gm. of food without the addition of salts supplied 0.020 gm. Ca and 0.245 gm. P.

[3] An approximation, due to spilling.

furnish a quantity of calcium equivalent to that present in 2 gm. of the unaltered salt mixture; with both 1 and 2% levels, the added calcium was in sufficient quantity to produce mixtures equivalent in calcium content to 4 and to 5 gm. of Osborne and Mendel salts, respectively. In each case, comparisons were made with amounts of unsupplemented salt mixture of equal calcium content. The feeding procedure, methods of analysis, and criteria were as described in the preceding paper. The data are shown in table 1.

In general, the bone ash obtained by the use of the supplemented salts was of the same order of magnitude as that observed when the unsupplemented Osborne and Mendel salt mixture of equal calcium content was used. When Osborne and Mendel salt mixture formed 0.5% of the diet, growth was markedly subnormal and calcification of the femora (40.3%) was below that of animals at weaning (42.8% at 21 days). With calcium added to the level in 2 gm. of Osborne and Mendel salts, there was a distinct acceleration in growth and an increase in the percentage of bone ash to the amount characteristic of that produced when 2% of the unaltered salt mixture was used. When either 1 or 2% Osborne and Mendel salts was supplemented with calcium carbonate as described, the same trend was observed, and in addition, the bone ash was within the limits desired in a rat of 200 gm. body weight and approximately 50 days of age, namely 58 to 60%. It is evident from the comparisons recorded in table 1 that any of the combinations that supply as much calcium as 4 or 5% Osborne and Mendel salts should be satisfactory. The new salt mixture has, therefore, been devised on this basis and the other constituents are furnished in the amounts present in 1 to 2 gm. of the same mixture. As a measure of safety, the trace elements, including iron, have been supplied at a level approximately that in 4% Osborne and Mendel salts. Although there is enough copper present as a contaminant in most diets, this element has been included in the new mixture in order to provide for studies in which a high degree of purification of the ingredients of the diet might remove too large an amount of copper. The composition of the new salt mixture is given in table 2.

To prepare the salt mixture the first seven salts are pulverized and mixed thoroughly. Suitable quantities of separately prepared standard solutions of the remaining constituents are then added and, after a thorough mixing of all ingredients, the product is dried in an air drier for 6 to 8 hours at 80 to 85° and then ground in a mill to pass a 50-mesh sieve. It is possible, however, to make a satisfactory product without the

addition of aqueous solutions and the subsequent drying. The calculated composition of salt mixture 351 and of Osborne and Mendel salts is given in table 3. In the experiments that follow the new mixture has been used without the added copper, inasmuch as none is supplied in the original Osborne and Mendel salts.

TABLE 2

Salt mixture 351

	gm.		gm.
$CaCO_3$	543.0	$FePO_4.4H_2O$	20.5
$MgCO_3$	25.0	KI	0.08
$MgSO_4$	16.0	$MnSO_4$	0.35
NaCl	69.0	NaF	1.00
KCl	112.0	$Al_2(SO_4)_3K_2SO_4$	0.17
KH_2PO_4	212.0	$CuSO_4$	0.90

Figures are for 'reagent' or 'e.p.' salts.

TABLE 3

Calculated composition of salt mixtures

	351 [1]	OSBORNE-MENDEL [2]		351 [1]	OSBORNE-MENDEL [2]
Calcium	0.435	0.462	Iron	0.0103	0.0112
Magnesium	0.0209	0.0597	Iodine	0.000122	0.000131
Sodium	0.0554	0.128	Manganese	0.000254	0.000246
Potassium	0.239	0.684	Fluorine	0.000904	0.000961
Phosphorus	0.102	0.279	Aluminum	0.0000194	0.0000219
Chlorine	0.191	0.444	Copper	0.000716	
Sulfur	0.00908	0.0259			

Data are in grams contained in 2 gm. 351 salt mixture and in 4 gm. Osborne and Mendel salt mixture, the quantities usually included in 100 gm. of complete diet.

[1] Figures are for the salt mixture dried at 80 to 85° for 6 to 8 hours.

[2] Due to moisture these figures are lower than the theoretical values. They represent the composition of the salt mixture as used in these experiments.

The new mixture has been in use in this laboratory for about 2 years and has been compared with the Osborne and Mendel salt mixture under various experimental conditions. In order to subject the new preparation to a severe test, these comparisons were made when only 2% was incorporated in the food. Reference to table 3 shows that if this amount is supplied, the calcium content of the food is somewhat below

that with 4% Osborne and Mendel salts. There are some indications that 2% 351 salts may be near the lower limit of safety, particularly for short time experiments with very rapidly growing animals, and it is suggested that the amount be increased to 2.5% in such cases.

For the comparisons of the two salts, the food combinations summarized in table 4 were used. The diets were modifications of the mixture of purified foodstuffs described in the preceding paper. In all cases, except where noted, the general conduct of each experiment was as previously described.

TABLE 4
Percentage composition of diets[1]

DIET NO.	1	2	3	4	5	6	7	8	9[2]	10[2]
Casein	18	12	50	18	18	18				18
Lactalbumin							18	1.5		
Gliadin									18	
Butter fat	9	9	9	9		9	9	9	9	9
Lard	20	20	20	20		54	20	20	20	20
Starch	43	49	11		72	9	42–43	59.5	51	51
Dextrin				43						
351 salts	2	2	2	2	2	2	2–3	2	2	2
Yeast	8	8	8	8	8	8	8	8		

[1] Control diets for all series contained 4% Osborne and Mendel salt mixture with a corresponding reduction in carbohydrate.

Animals in all except series 5 received 10 drops of cod liver oil daily. Those in series 5 received 2 drops per week of a cod liver oil concentrate.

[2] Animals in series 9 and 10 received 400 mg. of yeast daily.

Table 5 presents data obtained with 12, 18 or 50% casein in the diet, 18% lactalbumin, a diet that contained no added fat other than that incidental to the fat-soluble vitamins employed, and one in which dextrin was used in place of starch.

The first comparisons, those of the animals fed 18% casein diets, represent larger numbers of animals than any of the other groups, inasmuch as positive controls of this type have been maintained at all times, in order to detect possible variations in the animals of the colony. By this means, every experiment has been controlled by the use of the standard

diet and with both salt mixtures. The average bone ash for the fifty-two control animals fed 2% 351 salts falls somewhat below that for the thirty-nine fed 4% Osborne and Mendel salt mixture, being 58.6% in contrast to 60.2%. This is due in part, however, to a difference in rate of growth, the average

TABLE 5

Comparison of salt mixture 351 and Osborne and Mendel salt mixture under different dietary conditions[1]

DIET NO.	TYPE OF DIET	SALTS IN DIET		DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE	TOTAL P INTAKE	ASH OF DRY FAT-FREE FEMURS
		Type	Per cent					
				gm.	gm.	gm.	gm.	%
1	18% casein	351	2	5.4	295	1.34	1.02	58.6
		351	2.5	5.4	286	1.64	1.07	60.2
		0–M	4	4.8	322	1.56	1.69	60.2
2	12% casein	351	2	5.0	355	1.62	0.94	59.6
		0–M	4	4.8	360	1.74	1.60	60.3
3	50% casein	351	2	5.0	283	1.39	2.21	57.9
		0–M	4	5.2	269	1.40	2.58	58.7
4	Dextrin	351	2	6.1	262	1.19	0.91	57.5
		0–M	4	5.4	277	1.34	1.45	58.1
5	Low fat	351	2	5.4	409	1.86	1.42	59.7
		0–M	4	5.8	385	1.86	2.02	59.4
7	18% lactalbumin	351	2	4.5	321	1.40	0.33	56.8
		351	3	5.3	284	1.85	0.43	58.1
		0–M	4	5.0	297	1.38	0.83	60.0

[1] With the control diet (18% casein) fifty-two rats were fed 2% 351 salts and thirty-nine rats were fed 4% Osborne and Mendel salts. Ten rats were used in each of the other groups.

for the animals of the first series being 5.4 gm. a day in contrast to 4.8 [2] gm. a day for the second. The average total food intake was considerably lower than 351 salts were used. With 2.5% of the new salt mixture, growth was at the same rate as with 2%, and the bone ash, 60.2%, was equal to that produced with 4% Osborne and Mendel salts.

[2] The figure for rate of growth, 4.8 gm. a day, is lower than that recorded in the preceding paper. It is an average of thirty-nine rats instead of the ten previously reported.

That the lower average bone ash for the animals fed 2% 351 salts is due in part to more rapid growth and the accompanying lowered food intake is seen in the results of a carefully controlled study of two groups of animals, in which the factor of variable food intake was eliminated. To make this comparison, litter mates of the same weight were selected and, in pairs, were fed the standard ration with either 2% 351 salts or 4% Osborne and Mendel salts. Unlike the usual procedure in paired feeding experiments, each animal was allowed to consume food ad libitum. Comparisons were made on the basis of equal food intake, the amount for a given pair being determined by that consumed by the first member of the pair to reach 200 gm. body weight. The second animal was killed when it had eaten the same amount of food. Of fourteen such pairs, three were discarded because the final weights were too divergent for satisfactory comparisons. The data are given in table 6.

Under the conditions of this experiment, there is very close agreement in bone ash for the two members of each pair. Although the average bone ash with salt mixture 351 as reported in table 5 is lower than when the Osborne and Mendel salt mixture was used, with these paired rats of the same initial and final weight and same food intake this difference is not observed. In spite of wide variations in rate of growth and bone ash for each salt mixture, the averages are the same, a gain of 5.3 gm. a day and a bone ash of 58.4%. The average total food intake of 290 gm. is of the same order of magnitude as that for the fifty-two control rats on 2% salt mixture 351 (table 5) and supplies further evidence that the slightly higher bone ash of rats fed Osborne and Mendel salts is due in part to an increased food intake.

It is not the purpose of this report to compare one ration with another, but merely to determine whether the new salt mixture can replace the old under various dietary conditions. On this basis we find comparable results in most cases. With some of the less common diets, such as that containing 12% casein, that with 50% casein, and the low fat diet, we find the

general type of results that would be anticipated. With both the 12% casein and the low fat diets there was greatly increased food consumption, and in both series the bone ash was approximately 60% with each salt mixture. With 351 salts

TABLE 6

Comparison of salt mixture 351 and Osborne and Mendel salt mixture under conditions of equal food intake

RAT NO.	SALTS IN DIET		DAILY GAIN	FINAL WEIGHT	TOTAL FOOD INTAKE	TOTAL Ca INTAKE	TOTAL P INTAKE	ASH OF DRY FAT-FREE FEMURS
	Type	Per cent						
			gm.	gm.	gm.	gm.	gm.	%
C7824	351	2	5.0	194	277	1.26	0.96	59.5
C7825	0–M	4	6.6	200	277	1.34	1.45	58.3
C7832	351	2	5.0	200	302	1.37	1.05	59.6
C7833	0–M	4	5.0	194	302	1.46	1.59	59.2
C7830	351	2	5.6	200	283	1.29	0.98	57.8
C7831	0–M	4	5.0	194	283	1.37	1.49	57.6
C7933	351	2	5.0	200	280	1.27	0.97	59.3
C7930	0–M	4	4.8	194	280	1.36	1.47	59.2
C7943	351	2	5.4	200	310	1.41	1.08	59.8
C7942	0–M	4	5.0	196	310	1.50	1.63	59.6
C8039	351	2	6.1	200	275	1.24	0.94	56.1
C8038	0–M	4	6.6	200	275	1.32	1.43	57.6
C8040	351	2	5.8	200	277	1.26	0.96	58.8
C8041	0–M	4	5.6	200	277	1.34	1.45	57.0
C8046	351	2	5.0	200	301	1.36	1.04	61.0
C8045	0–M	4	4.7	190	301	1.45	1.58	60.6
C8127	351	2	6.1	200	281	1.27	0.97	56.3
C8128	0–M	4	4.4	192	281	1.36	1.47	57.0
C8136	351	2	5.6	200	295	1.34	1.02	59.3
C8137	0–M	4	5.4	196	295	1.43	1.55	57.1
C8153	351	2	4.1	200	322	1.47	1.12	60.5
C8151	0–M	4	5.0	200	322	1.56	1.69	59.6
Average	351	2	5.3	199	290	1.32	1.01	58.9
Average	0–M	4	5.3	196	290	1.41	1.53	58.4

and 12% casein, growth was slightly below that of the control animals; with Osborne and Mendel salts there was no change in the growth rate as a result of the lower intake of protein. With the low fat diet and 351 salts, growth was normal (5.3

gm. a day) and with Osborne and Mendel salts it was decidedly above normal (5.8 gm. a day in contrast to 4.8 for the control diet). The intake of calcium and phosphorus was high in both cases and consequently good bone was produced in spite of the rapid growth. If the casein content of the diet was increased to 50%, food intake was low with both salt mixtures and the agreement in degree of calcification was good.

The experiment with lactalbumin as the protein was designed particularly to test the adequacy of the phosphorus supply in salt mixture 351. Although most rations in which a salt mixture is used would be likely to supply additional phosphorus, usually in the form of casein, it seemed desirable to determine what type of growth and what sort of bone would be produced when the only source of phosphorus was that found in the salt mixture itself. The mixture was supplied at two levels, the usual 2% and also at 3%. With 2% salts, growth was slow, being only 4.5 gm. a day, and the bone ash was significantly below that obtained when 4% Osborne and Mendel salts was used, being 56.8% in contrast to 60.0%; when the proportion of salt mixture was increased to 3% of the diet, growth was more rapid, being 5.3 gm. a day, and the bone ash was 58.1%. This figure for bone ash is still somewhat lower than that obtained with 4% Osborne and Mendel salts and lactalbumin as the protein. However, it is comparable with that produced when 2% 351 salts is used with an 18% casein food, and the rate of growth is practically the same. On the basis of this observation, it is suggested that 3% 351 salts be used with rations that supply no other phosphorus than that in the salt mixture.

Dextrin is frequently used to replace starch as a source of carbohydrate. When this substitution was made growth was rapid with low food intake and the bone ash was slightly below the desired 58 to 60% with both salt mixtures.

Although the main object of the experiments summarized in table 5 has been to demonstrate the adequacy of the new mixture as a substitute for the old, the comparisons that have been made have, in addition, furnished data concerning the

calcium and phosphorus intake of animals during the period of active growth from 60 to 200 gm. body weight.

The total and daily average intakes of calcium and phosphorus have been calculated for those groups in which the average rate of gain was in excess of 5 gm. a day and the bone ash was above 58%. With salt mixture 351, the average daily calcium intake varied from 44 to 59 mg., and the phosphorus from 13 to 45 mg. In the case of the Osborne and Mendel salts, the calcium intake was 42 to 64 mg. and the phosphorus 26 to 80 mg. per day. It is obvious that the intake of phosphorus has varied within wide limits without affecting the rate of growth or percentage of bone ash.

The individual records of the animals in the two control groups, namely those with either 2% 351 salts or 4% Osborne and Mendel salts in a diet containing 18% casein, have been studied in detail. With the new salt mixture, 75% of the animals gained in excess of 5 gm. a day on an average daily intake of 47 mg. calcium and 36 mg. phosphorus. With the Osborne and Mendel salt mixture, 54% of the rats gained at the same rate and the average daily calcium and phosphorus intakes were 55 and 60 mg., respectively. Inasmuch as an increase in the amount of 351 salts in the diet from 2% to 2.5% furnished very little more phosphorus and permitted the production of 60.2% bone ash, with no decrease in rate of growth (5.4 gm. a day), it would seem that an average phosphorus intake of 60 mg. per day was unnecessarily high under the conditions of this experiment.

Many nutrition investigations involve a limitation in the rate of growth by artificial means, or so-called 'stunting.' The two salt mixtures have accordingly been compared under certain of these conditions. These experiments, summarized in table 7, differ from those already described in that the rations were supplied for definite periods of time, since, under the conditions of feeding, the animals could not be expected to reach 200 gm. at a rate that would make comparisons significant. In the first series, the 'calorie control,' in which the standard rations were fed in suboptimal amounts, the period

of growth was 30 days for one group and 60 for another. The object of the longer period was to determine whether, with the low daily intake of calcium and phosphorus imposed by the restricted amount of food, there would be adequate calcification if the food was supplied over a sufficiently long period of time. Comparison of the two series shows that this is true, and also that differences in calcification, evident

TABLE 7

Comparison of salt mixture 351 and Osborne and Mendel salt mixture when growth is restricted[1]

DIET NO.	TYPE OF DIET	SALTS IN DIET		TIME ON DIET	FINAL WEIGHT	DAILY GAIN	TOTAL FOOD INTAKE	TOTAL Ca INTAKE	TOTAL P INTAKE	ASH OF DRY FAT-FREE FEMURS
		Type	Per cent							
				days	*gm.*	*gm.*	*gm.*	*gm.*	*gm.*	%
1	Calorie control	351	2	30	111	2.1	150	0.68	0.52	54.6
		0–M	4	30	111	2.2	153	0.74	0.80	56.0
1	Calorie control	351	2	60	158	1.8	300	1.37	1.04	59.2
		0–M	4	60	155	1.8	306	1.48	1.61	59.9
8	1.5% lactalbumin	351	2	40	73	0.6	284	1.24	0.29	59.0
		0–M	4	40	74	0.7	245	1.14	0.69	59.8
6	High fat	351	2	30	127	2.7	183	0.83	0.64	53.7
		0–M	4	30	118	2.4	173	0.84	0.91	55.3
9	18% gliadin	351	2	30	68	0.6	131	0.60	0.13	57.6
		0–M	4	30	67	0.6	122	0.59	0.34	57.6
9	18% gliadin	351	2	30	71	0.8	150	0.68	0.15	
10	18% casein			30	200	4.3	290	1.32	1.01	62.1
9	18% gliadin	0–M	4	30	70	0.7	144	0.70	0.40	
10	18% casein			33	200	4.1	287	1.39	1.51	63.2

[1] In each of three groups, the 30-day calorie control, 1.5% lactalbumin, and the high fat, ten rats were used. Five rats were used in each of the other groups.

at 30 days, have almost disappeared at 60. For the 30-day period, the total intake of calcium is low, 680 mg. with salt mixture 351 and 740 mg. with Osborne and Mendel salts, yet with the slow growth characteristic of animals on a restricted food intake, calcification is comparatively good. Animals permitted to reach the same body weight when the rations were supplied ad libitum showed slightly lower bone ash with

both salt mixtures, namely 52.9 and 54.7% for the 351 salts and the Osborne and Mendel salts, respectively, as compared with 54.7 and 56.0% for the slowly growing animals with restricted food intake.

When an adequate protein was supplied in very small amount, 1.5% lactalbumin, for a period of 40 days after weaning, growth was almost negligible and the animals did not even double their weight in that time. The total intake of calcium and phosphorus was low, but evidently sufficient for the small bones of the stunted animals, for the percentage of ash is high with both salt mixtures. The bone ash for the much younger control rats of the same body weight is between 46 and 47% in contrast to 59% or over when growth was restricted by this means.

If growth was limited by the use of a diet high in fat, the same type of result, both in rate of gain and percentage of bone ash, was obtained as when the intake of the standard ration was kept subnormal. Though the animals were permitted to consume food ad libitum, the intake was small, being only 10 to 15% above that consumed by the calorie restricted animals.

Retardation of growth due to the use of an incomplete protein, gliadin, resulted in a rate of gain that was very low. At the end of 30 days, calcification was relatively high for the body weight attained, being 57.6% for both salt mixtures in contrast to 47% for the controls at the same body weight. In another series a 30-day period of restricted growth with gliadin as the protein was followed by realimentation with a diet containing casein. The animals were permitted to reach the usual body weight of 200 gm. and calcification at the end of the total growing period of approximately 60 days was excellent in both cases.

SUMMARY

A new salt mixture has been described in which the proportion of calcium to other constituents has been greatly increased. This preparation, made by mixing dry salts, may be used at the level of 2% in many diets of purified foods which usually require 4% Osborne and Mendel salts.

In experiments designed to compare the two salt mixtures during a selected period of active growth, from 60 to 200 gm. body weight, at a rate in excess of 5 gm. a day, adequate calcification of the femora has resulted with an average daily intake of approximately 50 mg. calcium and 35 mg. phosphorus.

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THE EFFECT OF EXERCISE ON METABOLISM FOLLOWING THE INGESTION OF WATER, GLUCOSE AND FRUCTOSE, AS SHOWN BY THE COURSE OF THE RESPIRATORY QUOTIENT

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TWO FIGURES

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In a recent study (Bachmann and Haldi, '37) a rise in blood lactic acid and a fall in blood CO_2 were observed within 30 minutes after the ingestion of fructose, but not after glucose. Respiratory quotients after fructose, corrected for non-metabolic CO_2 in the expired air, in some instances remained above unity, indicating formation of fat; after glucose the quotients never reached unity. Since exercise increases the proportion of carbohydrate oxidized, these differences in the metabolism of glucose and of fructose raise several interesting questions: If glucose or fructose were ingested immediately before exercise, would a greater part of the energy of exercise be supplied by carbohydrate than when no sugar is taken? Would both sugars serve equally well to spare the carbohydrate stores of the body? Would exercise have an accelerating effect on the metabolism of either or both these sugars, with a greater utilization of the ingested sugar during exercise than at rest?

An attempt to answer these questions has been made in the present study of the respiratory exchange after the ingestion of glucose and of fructose, both with the subject at

rest and taking exercise immediately after ingestion of the sugar. Although a similar study was reported several years ago by Carpenter and Fox ('31 b, '31 c) we believed the question to be of sufficient importance to warrant further investigation.

METHOD

The respiratory exchange was determined by the open circuit method of Carpenter and Fox ('31 a). The gas meter was read at 15-minute intervals; the gas samples collected in the manner described elsewhere (Bachmann and Haldi, '37), were analyzed by two analysts using the Haldane-Henderson method. The duplicate analyses were required to check within 0.02%.

The subjects of the experiments were two male adults, one (J. H.) 38 years of age, 182 cm. in height, weighing 80 kilos, the other (W. W.) 27 years of age, 170 cm. in height, weighing 56 kilos. All the experiments were conducted at approximately the same time of day, from 7.30 to 11.00, the last meal having been taken not later than 7 o'clock the evening before. On the morning of the experiment the subject rode to the laboratory and then reclined for 30 minutes or more on a couch placed near the respiration apparatus.

After the preliminary rest the respiratory exchange was determined for three consecutive 15-minute post-absorptive or basal periods. The subject then arose, voided urine and ingested either 500 cc. of water alone at 37°C. or 50 gm. of glucose or fructose, or 25 gm. of each in the same amount of water. The control experiments with water alone were suggested by those of Carpenter and Fox ('30) which showed a measurable increase in metabolism after 500 cc. water. Two minutes after the beginning of the ingestion, a signal was given for the resumption of the experiment.

Two types of experiments were conducted: One, with the subject remaining at rest throughout seven 15-minute periods after ingestion; the other, with the subject taking exercise on a Prony brake bicycle ergometer for two consecutive 15-minute

periods immediately after the ingestion, and then reclining for five 15-minute periods. The exercise experiments were preceded by a 3 weeks period of training during which the same amount of work was done on 5 or 6 days a week as in each of the experiments.

In the rest experiments the subject reclined on the couch continuously except for 2 or 3 minutes at the end of the post-absorptive periods when he arose to void urine and ingest the water or sugar solution. In the exercise experiments, he mounted the bicycle immediately after the ingestion, inserted the mouthpiece and at a given signal began the exercise. The tension on the ergometer wheel was maintained constant at 2.5 kg. and the rate of pedalling timed to the beat of a metronome. Work was done at approximately 550 kilogrammeters per minute. Upon the completion of the exercise, the subject 'rolled off' the bicycle on to the couch without interrupting the experiment. Whenever it appeared from the kymograph record that the subject was about to go to sleep a buzzer was sounded, to which he responded with the same signal. At the end of the experiment the urine was again collected. The total nitrogen of the urine samples was determined for calculating the non-protein respiratory quotients.

RESULTS AND INTERPRETATION

For the sake of clarity a brief description of the respiratory quotients in the rest experiments will be given first, followed in order by a description and interpretation of the quotients of 1) the two exercise periods, 2) the first recovery period, 3) the last four recovery periods. The non-protein respiratory quotients hereafter referred to as the 'respiratory quotients,' are shown for the various groups of rest and exercise experiments in figure 1, each curve representing an average of at least three experiments. The average of all the post-absorptive quotients has been taken as a common base line. Figure 2 facilitates a comparison of the increment in the respiratory quotient during any period of the various groups of experiments. It will be observed from the curves

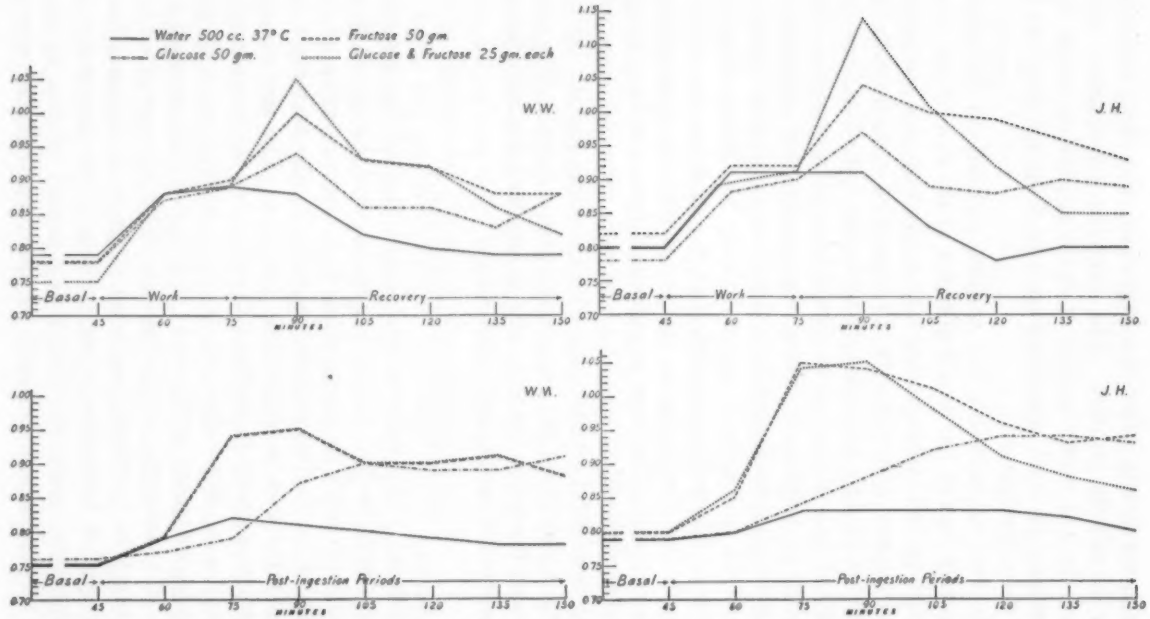

Fig. 1 The course of the respiratory quotient in two subjects (W. W. and J. H.) following the ingestion of water, glucose, fructose and a mixture of these two sugars when at rest (lower curves), and during exercise and recovery (upper curves).

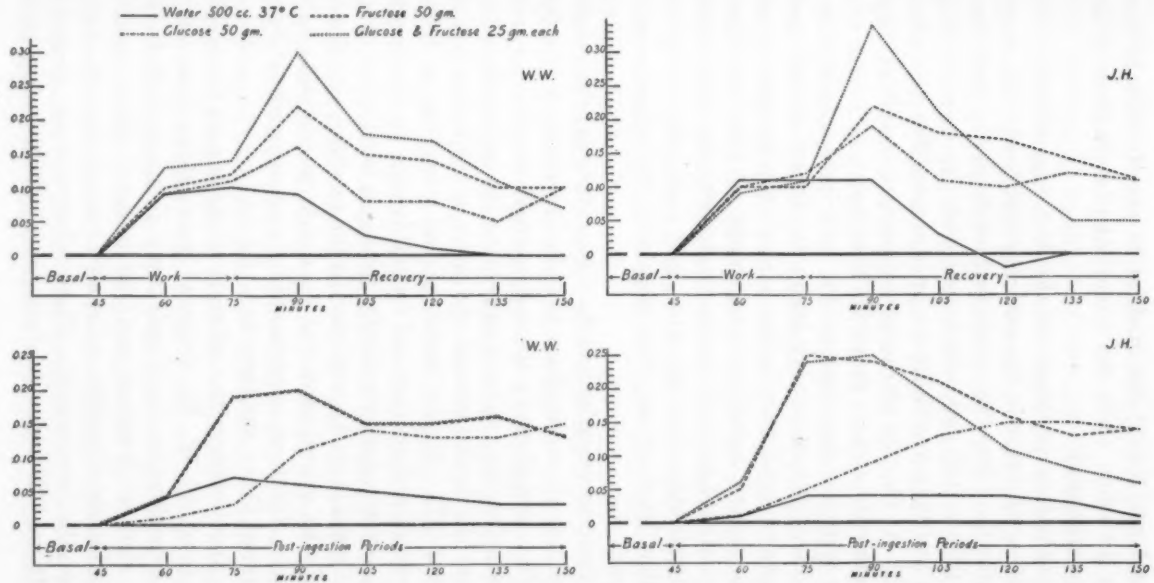

Fig. 2 Increments of the respiratory quotient above the base line in two subjects following the ingestion of water, glucose, fructose and a mixture of these two sugars when at rest (lower curves), and during exercise and recovery (upper curves).

in figures 1 and 2 that the qualitative results with both subjects are the same while the quantitative data show a fairly close approximation.

The respiratory quotients of the rest experiments. After the ingestion of water in the control experiments the respiratory quotients of the two subjects rose from the base line of 0.76 and 0.79 to 0.82 and 0.83, respectively, an increase of 0.06 and 0.04, and then gradually dropped toward the post-absorptive level. At the end of the experiment the quotient of each subject was still slightly above the base line.

In the glucose experiments the respiratory quotient remained practically stationary during the first 15-minute period after ingestion, and then in the next 15-minute period began a gradual ascent, reaching a maximum of 0.90 and 0.94 an increment of 0.14 and 0.15, respectively, with the two subjects, 45 and 60 minutes after ingestion, and continuing at approximately this level during the remaining 30 minutes of the experiment.

In the fructose experiments the respiratory quotient showed a distinct rise above the base line during the first 15-minute period after ingestion; in the next period the rise was more abrupt bringing the quotient almost to the maximum of 0.95 with one subject and 1.05 with the other, a respective increase of 0.20 and 0.25. In the fourth period there was a drop in the quotient of each subject and from this time on, the quotient of W. W. remained at practically the same level while that of J. H. continued in its descent toward the baseline. At the conclusion of the experiment, the respiratory quotients in the glucose and fructose experiments were approximately the same distance above the base line (fig. 1).

In the experiments with a mixture of the sugars the respiratory quotient of W. W. followed throughout that of the fructose experiments; the respiratory quotient of J. H. also followed that of fructose until 60 minutes after ingestion when it fell more abruptly and came closer to the base line at the end of the experiment. These observations agree with those of Deuel ('27) who obtained practically the same results

from 75 gm. fructose as from a 75 gm. mixture of fructose and glucose in equal parts.

The respiratory quotient of exercise. During the first 15-minute exercise period there was a marked rise in the respiratory quotient in all the experiments. With subject W. W. it attained approximately the same level in the water as in the sugar experiments, whereas there was some variation with subject J. H. The actual rise above the base line, however, as seen in figure 2 was approximately 0.10 in all the experiments except in those on W. W. with a mixture of the sugars. The respiratory quotient was usually slightly higher in the second than in the first 15-minute exercise period.

Interpretation. In the control experiments there was an increase in both the absolute and relative amount of carbohydrate oxidized. Whereas carbohydrate supplied only about 25% of the total energy during the post-absorptive periods, approximately 65% was derived from this foodstuff during exercise.

The percentage of carbohydrate oxidized as shown by the respiratory quotient was not increased by the ingestion of the sugars immediately before exercise. It is worthy of note that the ingested glucose and fructose were equally ineffective in this respect. The experiments on W. W. with a mixture of the sugars in figure 2 are somewhat irregular. As this irregularity did not occur in the absolute respiratory quotient (fig. 1), we believe that it should be attributed to an unusually low post-absorptive quotient which was obtained in these experiments and not to a specific effect of the mixture of the sugars. The average post-absorptive quotient was only 0.75 as contrasted with quotients of 0.79, 0.78 and 0.78 obtained in the water, glucose, and fructose experiments, respectively. We are at a loss to account for the unusually low quotient.

The total amount as well as the percentage of carbohydrate oxidized during exercise was unaffected by the previous ingestion of the sugars. The total amount metabolized by W. W. during the two 15-minute exercise periods was 29, 29, 29 and 30 gm. in the experiments with water, glucose, fructose

and a mixture of the sugars, respectively, and by J. H. 32, 32, 33, and 33 gm., respectively. The average difference of 1 to 2 gm. for the ½ hour of exercise is regarded as of little or no significance, since the same variation was observed in individual experiments of the control series.

It is obvious from figure 2 that the rise in the respiratory quotients produced by the sugars themselves as shown in corresponding periods of the rest experiments, was not superposed upon the rise induced by exercise. On casual examination it may appear as if the metabolism of the ingested sugars was inhibited by exercise. This apparent inhibition is especially striking in the case of fructose and the mixture of glucose and fructose. It can be shown from analysis of the data, however, that such a conclusion is unjustified. In the experiments with fructose, for example, the changes in the oxygen consumption and carbon dioxide elimination were sufficient to produce a marked increase in the respiratory quotient in the rest experiments. They were so small, however, relative to the oxygen consumption and carbon dioxide elimination during exercise, that when added to the latter they had no appreciable effect on the respiratory quotient. It is reasonable to assume that the metabolism of the ingested sugars proceeded to at least as great an extent during exercise as in the corresponding periods of the rest experiments, thereby serving to spare the body stores of carbohydrate.

The respiratory quotient of the first 15-minute recovery period. The respiratory quotient during the first recovery period in the control experiments remained at practically the same level as during exercise. In the experiments with the sugars it rose well above the exercise level. The greatest rise occurred in the experiments with a mixture of the sugars and the smallest in the glucose experiments. With the mixture the quotient of each subject went above unity (fig. 1) while with fructose that of one subject rose to unity and that of the other above unity; with glucose the highest average quotient was 0.94 for one subject and 0.97 for the other.

Interpretation. The increase in carbohydrate metabolism induced by exercise as shown in the control experiments persisted for a few minutes after exercise. The interpretation of the first recovery period of the sugar experiments presents some difficulties; first, because of the marked rise of the quotients during this period above that of the exercise level, and secondly, because of the greater rise with the mixture of the sugars than with glucose or fructose alone. As regards the rise above the exercise level, the possibility that it was due to lactic acid formation with the consequent blowing off of non-metabolic carbon dioxide should first be considered, because of the increase in blood lactic acid observed after the ingestion of fructose (Campbell and Maltby, '28; Bachmann and Haldi, '37). There are reasons, however, militating against this possibility: 1) While there was no appreciable difference in the amount of lactic acid formed from 50 gm. fructose or a mixture of 25 gm. glucose and 25 gm. fructose, in the present experiments there was a considerably greater increase in the respiratory quotient of the first recovery period in the experiments with a mixture of the sugars than with fructose; 2) the large rise in the quotient during the first recovery period in the experiments with fructose or a mixture of the sugars, could not be accounted for by the liberation of non-metabolic carbon dioxide, even though it be assumed that the maximum amount of lactic acid found in the experiments quoted above, was formed in that period.

There is the possibility that the metabolism of the sugars may have been superposed upon the normal metabolism of recovery. A quantitative analysis has accordingly been made of the data on the oxygen consumption and carbon dioxide elimination of the first recovery period in the exercise experiments and of the corresponding period in the rest experiments. The increase in oxygen consumption and carbon dioxide elimination due to the sugars in the third period after ingestion in the rest experiments when added to the oxygen consumption and carbon dioxide elimination of the first recovery period in the control experiments (normal metabolism

of recovery) gave respiratory quotients lower than those actually obtained in the exercise experiments with the sugars. While the results are not conclusive, they are nevertheless highly suggestive. It would appear that the rise of the respiratory quotient above the exercise level in the first recovery period of the sugar experiments was due only in part to a superposition of the metabolism of the sugars upon the normal metabolism of recovery. The failure of the theoretical quotients to coincide more closely with the actual indicates that a change had been induced in the metabolism of the sugars during exercise which persisted during the first recovery period.

Further analysis of the data give suggestive results as regards the nature of this change. In the sugar experiments the average net excess oxygen consumed in the first recovery period was subtracted from the average net excess carbon dioxide produced to obtain what may be designated as the surplus carbon dioxide. The surplus carbon dioxide of the corresponding period of the rest experiments was obtained by the same procedure. Since in the oxidation of carbohydrate one volume of oxygen yields one volume of carbon dioxide, there should be no surplus carbon dioxide, if the excess oxygen were used exclusively for the oxidation of the ingested sugars. In the first recovery period of the exercise experiments with glucose, fructose and a mixture of the sugars, it was 250, 510 and 1000 cc., respectively, and in the corresponding periods of the rest experiments, 110, 480 and 430 cc., respectively. It will be observed that whereas the surplus carbon dioxide was the same in the rest and exercise experiments with fructose, it was greater in the exercise than in the rest experiments with glucose and a mixture of the sugars. These data suggest a transformation into fat of a part of the ingested glucose and fructose at rest and in the case of glucose an acceleration of the process during exercise, which continued during the first period of recovery. The acceleration may have been due perhaps to an increase in body temperature (compare Pembrey and Nicol, 1898). The larger

surplus carbon dioxide in the exercise experiments with a mixture of the sugars than with fructose would account for the greater rise in the respiratory quotient. An objection to this interpretation is found in the greater surplus of carbon dioxide in the experiments with a mixture of the sugars than in those with glucose. It is not at all impossible, however, that the metabolism of glucose under the influence of exercise may have been modified by the simultaneous metabolism of fructose.

The respiratory quotients of the last four recovery periods. In the control experiments the respiratory quotient of each subject fell abruptly during the second recovery period; in the next period, it declined practically to the base line where it remained to the end of the experiment.

In all the sugar experiments the respiratory quotient likewise fell abruptly during the second recovery period. From this time on the quotient remained almost constant in the glucose experiments, whereas it fell gradually toward the base line in those with fructose. In the experiments with a mixture of the sugars the fall was gradual with one subject and abrupt with the other, for three recovery periods; it then remained constant during the last 15-minute period. At the conclusion of the experiments the quotients were well above the base line in all the sugar experiments, being at the highest level with one subject with fructose, the lowest with a mixture of the sugars and at an intermediate position with glucose; with the other subject, the quotient of glucose ascended abruptly to the level of that of fructose. If this unusual rise had not occurred, it is likely that approximately the same relations would have prevailed as with the other subject. A quantitative comparison of the net increase in the respiratory quotients of the last four recovery periods with the corresponding periods of the rest experiments is presented in table 1. The net increase represents the difference between the increase above the post-absorptive level in the control and that in the sugar experiments.

Interpretation. The smaller net increase of the respiratory quotients in the exercise than in the rest experiments after glucose indicates that there was less of the ingested sugar

TABLE 1

Net increase in the respiratory quotient during the last four 15-minute recovery periods and in the corresponding periods of the rest experiments

SUGAR INGESTED	SUBJECT	TYPE OF EXPERIMENT	NET INCREASE [1]			
			(1)	(2)	(3)	(4)
50 gm. glucose	W. W.	Rest	0.09	0.09	0.10	0.12
		Exercise	0.05	0.07	0.05	0.10
	J. H.	Rest	0.09	0.11	0.12	0.13
		Exercise	0.08	0.08	0.12	0.11
50 gm. fructose	W. W.	Rest	0.10	0.11	0.13	0.10
		Exercise	0.12	0.13	0.10	0.10
	J. H.	Rest	0.17	0.12	0.10	0.13
		Exercise	0.15	0.15	0.14	0.11

[1] The net increase was obtained by subtracting the increase over the basal in the control experiments with water from the increase over the basal in the sugar experiments.

TABLE 2

Net increase in carbohydrate oxidized during the last four 15-minute recovery periods and in the corresponding periods of the rest experiments

SUGAR INGESTED	SUBJECT	TYPE OF EXPERIMENT	NET INCREASE,[1] GRAMS CARBOHYDRATE				
			(1)	(2)	(3)	(4)	Total
50 gm. glucose	W. W.	Rest	1.3	1.3	1.2	1.5	5.3
		Exercise	0.6	0.8	0.5	1.2	3.1
	J. H.	Rest	1.3	2.1	1.7	1.9	7.0
		Exercise	1.0	1.4	1.4	1.1	4.9
50 gm. fructose	W. W.	Rest	2.1	2.3	1.5	1.2	7.1
		Exercise	2.1	2.2	1.7	1.5	7.5
	J. H.	Rest	1.6	2.2	1.6	1.9	7.3
		Exercise	2.3	2.6	1.8	1.5	8.2

[1] The net increase was obtained by subtracting the increase over the basal in the control experiments with water from the increase over the basal in the sugar experiments.

available for metabolism during recovery than in the corresponding rest periods. It may therefore be concluded that the metabolism of the ingested glucose was accelerated by exercise.

In the fructose experiments there was no appreciable nor consistent difference between the net increase of the quotients in the exercise and rest experiments. The metabolism of the ingested fructose, therefore, was not accelerated during exercise. These observations on the two sugars agree with those of Carpenter and Fox ('31 c).

Calculations by the same procedure of the net increase in carbohydrate oxidized (table 2) show that in the glucose experiments the average amount of carbohydrate burned during the recovery periods was 2.2 gm. less than in the rest experiments. This confirms the conclusion drawn from the respiratory quotients that the metabolism of glucose was accelerated during exercise thereby reducing the amount available during recovery. In the experiments with fructose practically the same amount of carbohydrate was oxidized during recovery as in the corresponding time in the rest experiments.

DISCUSSION

The results of these experiments, as stated above, suggest a conversion of a portion of the ingested glucose into fat under resting conditions and an acceleration of this process by exercise. It would appear therefore as if the respiratory quotients obtained after the ingestion of glucose are not exclusively combustion quotients. The common assumption that the respiratory quotients after the ingestion of glucose are combustion quotients is probably based on the close agreement in heat production measured by direct and indirect calorimetry, as reported by Lusk ('15) following the ingestion of the sugar when dissolved in warm water. It should be noted, however, that in a subsequent study, Murlin and Lusk ('15) found in each of six experiments on the dog that the heat production after the administration of 70 gm. glucose was greater by indirect than by direct calorimetry. Cathcart and Markowitz ('27) from a statistical analysis of these data, found a significant difference between the heat production as determined by the two methods. While they recognized that the data are insufficient for satisfactory treatment by statistical methods, their analysis assumes greater significance in

view of the constancy with which a higher heat production was obtained by the indirect method. These results suggest that the respiratory quotients from which the heat production was calculated, were not exclusively combustion quotients.

The failure of most workers to obtain respiratory quotients above unity after the ingestion of glucose may be partly responsible for the assumption that the quotients are true combustion quotients. As aptly stated by Rapport ('30), a respiratory quotient of unity has been established as a sort of dead line which makes it seem as though the conversion into fat cannot take place unless this quotient is exceeded, although there is no theoretical reason why this should be the case. Benedict and Carpenter ('18) some years previously had also called attention to the inference frequently made that the formation of fat from carbohydrate can occur only when the respiratory quotient is above unity. These authors, like Rapport, recognized the fallacy of this assumption.

Positive evidence for the conversion of glucose into fat is found in some of the respiratory quotients reported in the literature. Benedict and Carpenter ('18) obtained one measured respiratory quotient of 1.01 for one 30-minute period and several quotients of 0.98 and over. Since the "non-protein respiratory quotients were generally two or three points higher than the measured quotients, all values of 0.98 or over would, strictly speaking, represent a non-protein respiratory quotient of unity." Tögel, Brezina and Durig ('13) obtained a quotient of 1.03 in man after the ingestion of 100 gm. of glucose and Hanriot (1892; cited by Rapport, '30) a quotient of 1.07 after the administration of 73 gm. of glucose. The last named author has also published much higher quotients after glucose, although their accuracy has been questioned. Lublin ('26) found that quotients of his non-diabetic subjects rose in some instances above unity after the ingestion of 100 gm. glucose. Respiratory quotients well above unity were obtained on the dog by Lusk ('15) after the administration of 50, 60 and 70 gm. glucose. Similar results were obtained by Murlin and Lusk ('15) and by Morgulis and Pratt ('13).

As regards the respiratory quotients after the ingestion of fructose, it was shown in a previous communication (Bachmann and Haldi, '37) that they are not exclusively combustion quotients. In view of this observation and the foregoing considerations with respect to glucose, it becomes obvious that calculations of the amount of carbohydrate metabolized, heat production and the specific dynamic action of the sugar, based on the respiratory quotient, would yield incorrect values. The error in heat production, however, would be small because of the slight change in the value of the caloric factor within the range of the possible error of the respiratory quotient.

Calculations of the amount of carbohydrate oxidized after the ingestion of the sugars in these experiments, as shown in table 2, were made nevertheless, since it is likely that an error of the same order occurred in both the rest and exercise experiments. The conclusions drawn from a comparison of the two sets of experiments are, therefore, probably substantially correct. It should be noted that these conclusions support those arrived at from a consideration of the course of the respiratory quotient.

The results of our experiments differ in some respects from those obtained by Carpenter and Fox ('31 b). During exercise of the same intensity and duration, the respiratory quotients of their subject rose considerably higher than those of our two subjects, in some instances exceeding unity. The average quotients for the two periods of work on their control experiments were 1.00 and 0.96, whereas the average in our experiments was 0.88 and 0.89 with one subject and 0.91 in both periods with the other subject. Our lower quotients may possibly be accounted for by a more robust physique of our subjects. The high quotients obtained by Carpenter and Fox, some of which were above unity, suggest an accumulation of lactic acid and hyperventilation during exercise. The drop in the quotient below the base line in the second recovery period and its gradual rise toward the base line throughout the remainder of the experiment, would therefore appear to have been due to a compensatory retention of carbon dioxide during

the liquidation of the oxygen debt. In our experiments the quotient fell gradually after the first recovery period until it reached the base line where it remained until the end of the experiment. Carpenter and Fox believe that the fall in quotient below the base line indicates that the effect of work was to exhaust the available carbohydrate. If this were the case, it is difficult to account for the gradual rise in the quotient toward the base line which they observed during recovery.

The rise in the respiratory quotients obtained in the first recovery period of our experiments with the sugars was not observed by Carpenter and Fox. This difference in results can be explained if we assume that hyperventilation occurred in their experiments for, although the quotient fell below the base line after exercise in their glucose and fructose experiments, it was nevertheless 0.07 and 0.10 higher than in their control experiments with water. Notwithstanding these differences in experimental results some of our conclusions are essentially in accord with theirs. It can be readily seen that conclusions based on the difference between the results obtained in their sugar and control experiments would not have been invalidated by the effects of hyperventilation if the hyperventilation were practically the same in all the experiments.

SUMMARY AND CONCLUSIONS

The respiratory quotient as affected by exercise taken immediately after the ingestion of 500 cc. water, 50 gm. glucose, 50 gm. fructose, and a mixture of 25 gm. each of these two sugars dissolved in 500 cc. water at 37°C. has been studied on two subjects. The exercise consisted of performing 550 kilogrammeters of work per minute for two consecutive 15-minute periods on a Prony brake bicycle ergometer.

In the control experiments with water, the respiratory quotient rose to approximately 0.90 during exercise, an increase of 0.10 above the base line. The quotient of one subject was slightly higher during the second 15-minute period, whereas that of the other remained the same.

The increase of the respiratory quotient during exercise was practically the same when the sugars were ingested as when water alone was taken immediately before the exercise.

The rise in the respiratory quotient during exercise in the experiments with water and the sugars shows that there was an increase in the relative and absolute amount of carbohydrate oxidized.

Since the same rise in the quotient was obtained in the experiments with the various sugars as in the control experiments with water, it is concluded that the percentage of carbohydrate oxidized was not increased by the ingestion of the sugars immediately before exercise. Glucose and fructose were equally ineffective in raising the percentage of carbohydrate oxidized during exercise.

Practically the same amount of carbohydrate was oxidized during exercise in the control and various sugar experiments. In all the experiments there was more carbohydrate oxidized in the second than in the first exercise period.

During the first recovery period in the control experiments with water the respiratory quotient remained at practically the same level as during exercise, indicating that the increase in carbohydrate metabolism induced by exercise persisted some few minutes afterward.

In the experiments with the sugars there was a rise in the quotient during the first recovery period above the level reached during exercise. The greatest rise occurred in the experiments with a mixture of the sugars, the least in those with glucose. This may be explained in part as a superposition of the metabolism of the sugars on the normal metabolism of recovery. The data have been interpreted as suggestive of the conversion of a portion of the glucose into fat and of an acceleration of this process by exercise.

Exercise accelerated the metabolism of glucose whereas it had no effect on that of fructose. Inasmuch as the ingested glucose did not increase the total carbohydrate oxidation during exercise, it must have had a greater sparing effect than fructose on the body stores of carbohydrate.

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VITAMIN E AND GROWTH[1]

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TWO FIGURES

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On several occasions it has been suggested that vitamin E promotes growth and general well-being in addition to preventing sterility in male and female rats.

Evans ('28) demonstrated an impairment in the late growth of animals reared on a vitamin E-deficient diet. At 150 to 200 days of age both male and female animals ceased to grow, while litter mate animals which had received or were then given wheat germ oil or sterol-free concentrates continued to grow normally or resumed growth. Since castrated males responded to the same treatment the stimulus to growth was not mediated through the gonads. The basal diet contained 22% lard and 2% cod liver oil.

Mason ('29) observed that lettuce induced a marked increase in early growth rate of male rats when added to a diet apparently deficient only in vitamin E. From his studies and from a review of the earlier work of others he concluded that vitamin E per se was not responsible for this growth stimulation.

More recently Blumberg ('35) reported a series of feeding trials in which animals on vitamin E-deficient diets ceased growing when they were younger and at much lower weight levels than those of Evans. The rations were fat-free except

[1] Presented in part before the thirtieth meeting of the American Society of Biological Chemists at Washington, March 28, 1936 (J. Biol. Chem., vol. 114, p. lxxvii, 1936).

for small amounts of ethyl linolate and of ethyl laurate and oleate used as solvents for carotene and irradiated ergosterol. The animals became lethargic and later almost helpless. Their miserable condition could be improved by providing daily 1 gm. of wheat germ or 2 gm. of egg yolk, less well by 10 gm. of fresh lettuce and doubtfully by butter fat (0.5 gm.) or pasteurized milk (25 cc.). The negative results secured with supplements of the other recognized accessories and the accepted presence of vitamin E in the successful supplements led to the conclusion that vitamin E or some unrecognized associated fat-soluble factor was required for normal early and middle growth as well as for late growth and fertility.

Since our experiments were first reported (Olcott and Mattill, '36), Emerson and Evans ('37),[2] using female rats, have completed experiments successfully duplicating Evans' results and conclude that "the defective growth in experiments involving vitamin E occurs only after the 4th month of life." They were thus not able to confirm Blumberg's results.

In the observations of Ringsted ('35) on mature animals which had been deprived of vitamin E from weaning, emphasis is laid upon a paresis of the hind quarters which appeared at 160 to 220 days of age and which was accompanied later by symmetrical denuding and a hypoaesthesia of legs and tail. Animals which had, for test purposes, received vitamin E in the form of wheat germ oil and in amounts adequate for a successful gestation, remained free from these symptoms; the animals which demonstrated them had been used for assaying other possible vitamin E containing foods, all of them giving negative results. Ringsted found the condition somewhat similar to that in weanling rats first described by Evans and Burr ('28) and since observed by others (Morelle, '31; Mason, '33; Olcott and Mattill, '34). It should be noted that his basal diet contained 15 or 20% of lard, oxidized until it was markedly rancid by aeration at 110° for 10 hours, in order to insure

[2] We are indebted to Doctors Emerson and Evans for the opportunity of reading their manuscript "The effect of vitamin E deficiency upon growth," before publication.

the absence of vitamins A and E. The former was supplied as a supplement but unfortunately the possible curative effect of a potent wheat germ oil on the paretic animals was not determined. He concluded that the origin of the paresis was a lack of vitamin E of long standing and very high degree.

In this laboratory the extent of vitamin E deficiency of the basal diet (diet E, table 1) given to the animals used for routine testing, has often been questioned. The animals are usually sleek and well nourished and show no early cessation of growth. The casein in this diet is of commercial grade; it is not extracted nor is the yeast. Lipid extracts of these failed to provide vitamin E in adequate amount for successful gestation, and young animals reared from weaning on this diet practically never demonstrate first litter fertility even though the diet is fed without any preliminary aging to produce incipient rancidity. On the other hand, cod liver oil and lard have appeared not to be invariably lacking in vitamin E (Simmonds, Becker and McCollum, '28; Nelson, Jones and Taylor, '28).

It seemed that an answer to these questions could be obtained by rearing animals on diets rigorously freed of natural fats, these being replaced by distilled mixed ethyl esters of the fatty acids from such fats, and the lipid accessories provided in the form of carotene, calciferol, and vitamin E concentrate. If female animals reared on such a diet, but without the vitamin E supplement, required no larger doses of concentrate to insure a complete gestation than those reared on the stock E-deficient diet, the equivalence of the two diets in this respect would be demonstrated. Also further information could be secured on the relation of vitamin E to growth.

EXPERIMENTAL

Rats at weaning (24 to 29 days old) were placed in individual screen bottomed cages, and given the diets outlined in table 1. The carotene, vitamin E, and cod liver oil concentrate supplements were administered by dropping the solutions directly into the animals' mouths. Ethyl linolate and wheat

TABLE 1

Diets

Diet A:	Sucrose	61.5
	Extracted casein [1]	20
	Extracted yeast [2]	10
	Salts [3]	4.5
	Ethyl esters (distilled) of hydrogenated cottonseed oil [4]	4
	Calciferol [5] 0.1 mg./kg. of diet, dissolved in ethyl esters	
	Supplements (twice weekly):	
	Carotene [6] 0.1 mg., dissolved in ethyl esters	
	Ethyl linolate 100 mg. (3 drops) [6]	

Diet B: Diet A, with a further supplement of 2 to 5 mg. of vitamin E concentrate [7] from cottonseed oil.

Diet C: Diet A, plus a vitamin E concentrate from palm oil.

Diet E: Vitamin E-deficient diet, used in routine assays (Olcott and Mattill, '34).

Sucrose	45.5	Yeast [8]	8
Lard	22	Salts	4.5
Casein [8]	18	Cod liver oil	2

Diet F: Diet A plus a concentrate of the unsaponifiable matter of cod liver oil. [9]

Diet G: Diet A plus 2 drops wheat germ oil twice a week. [10]

Diet H: Diet A except 20% lard in place of 4% ethyl esters and 16% sucrose.

Diet J: Diet A except 4% lard in place of 4% ethyl esters (no linolate supplement).

Diet K: Diet A but with 20% ethyl esters (replacing 16% sucrose).

Diet L: Diet A but with 16% of lard replacing 16% sucrose.

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[1] Extracted five times with alcohol by warming on steam bath with frequent shaking. Alcohol was removed by filtering through muslin tied around the neck of the flask. The residue was extracted five times with ether and was then spread on trays and dried for 1 to 1½ hours in current of warm air.

[2] Courtesy of Northwestern Yeast Company. Extracted five times with ether as described above.

[3] Hawk and Oser ('31), Science, vol. 74, p. 369.

[4] A hydrogenated cottonseed oil was refluxed for 24 hours with twice its volume of absolute alcohol containing 2 to 3% dry hydrogen chloride. After cooling, the mixture was diluted with water and the ester layer washed repeatedly, centrifuged from occluded water, dried, and distilled in vacuo. The esters distilled below 160° at 0.1 mm. Until incorporation in the diet, they were kept cold and under carbon dioxide.

[5] We are indebted to Dr. C. E. Bills, Mead Johnson and Company, for generous supplies of these supplements.

[6] Prepared from commercial linoleic acid (Kahlbaum) as described in note 4. This was later omitted since several control feeding experiments demonstrated that the ethyl esters of cottonseed oil contained sufficient linolate.

[7] Prepared as previously described (Olcott and Mattill, '34) from cottonseed or palm oil, and fed in quantities of 2 to 5 mg. as determined by assay, such that each of the two doses per week provided enough for a fertile mating in a mature animal on the stock E-deficient diet (E).

[8] Unextracted.

[9] Four milligrams of the unsaponifiable matter of cod liver oil fed twice a week dissolved in ethyl esters.

[10] Two drops twice a week, mixed with the diet. Kindly supplied by General Mills, Inc. The assays indicated that this dosage should produce fertile animals.

germ oil were fed in a small amount of the diet, the food cups being removed until the supplement was eaten. The animals were weighed and fed the supplements twice weekly. At the end of the experimental growth period, males were sacrificed; a smear from the epididymis was examined for motile sperm and the testes were weighed and examined histologically.[3] The females were mated either before or after transfer to the stock vitamin E-deficient diet, in order to determine their fertility. Those which were deficient in vitamin E as shown by a resorption gestation, were given adequate amounts of a concentrate at the time of their second mating.

RESULTS AND DISCUSSION

The growth curves for several groups of rats are shown in figures 1 and 2. It is obvious that the animals on the highly purified diet A did not show the early cessation of growth described by Blumberg. Indeed, they grew practically as well as the colony animals whose growth curves he used for comparison. However, from the age of about 2 months the rate of growth of the male animals became progressively slower than that of the males receiving vitamin E (fig. 2). At 225 gm., the maximum weight attained by Blumberg's animals, they were still gaining about 12 gm. per week and the plateau at which growth finally ceased was at 300 gm.

Since the female animals were nearly all tested for fertility the data on growth are limited. Up to the time of the first pregnancy (3½ months of age), however, the rates of growth of the control animals (diet A) and of those receiving supplements of vitamin E were so similar that they have not been included in the figures. The growth curves are practically superimposable, an observation in agreement with that of Emerson and Evans ('37).[4]

Supplementing diet A with ethyl linolate (to obviate the possibility of an unsaturated fatty acid deficiency) or with a

[3] We are deeply indebted to Prof. E. Witschi of the Department of Zoölogy for his help in the preparation and interpretation of the sections.

[4] See footnote 2 on page 306.

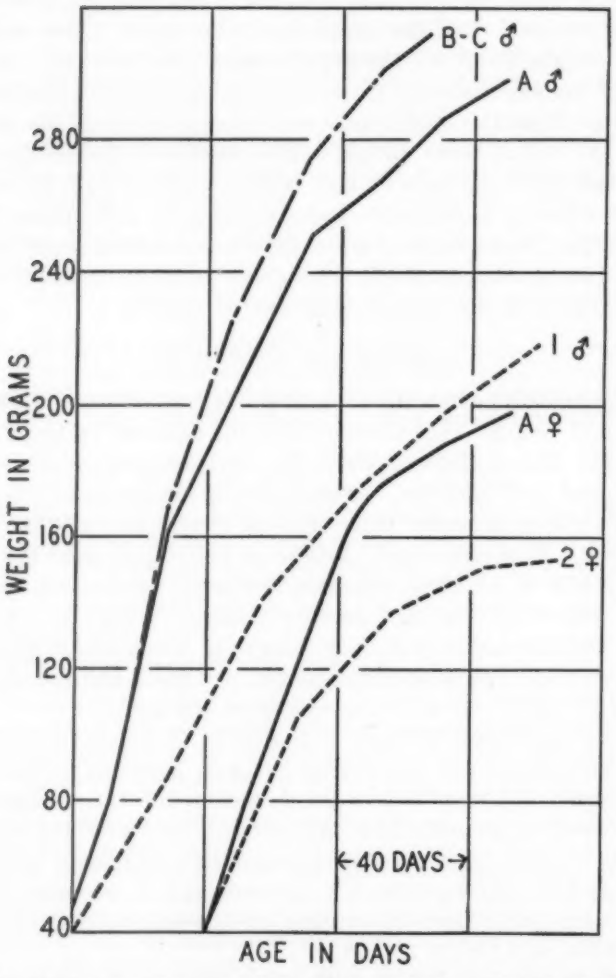

Fig. 1 Average growth curves of rats on (A) a highly purified vitamin E-free diet (twelve animals); (B–C) the same plus concentrates of vitamin E (twelve animals). In the females, vitamin E supplementation did not accelerate growth before the 110th day. (1) and (2) represent the growth curves obtained by Blumberg ('35).

cod liver oil concentrate (to augment the amounts of vitamins A and D furnished as carotene and calciferol) did not increase the growth rate or the maximum weight reached by the control animals.

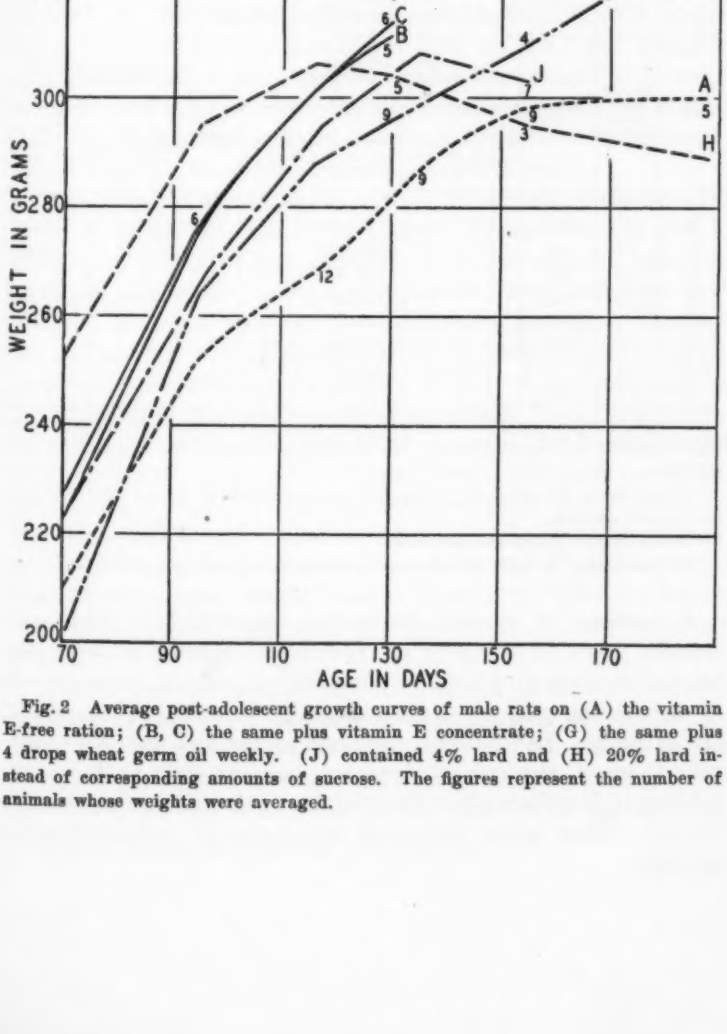

Fig. 2 Average post-adolescent growth curves of male rats on (A) the vitamin E-free ration; (B, C) the same plus vitamin E concentrate; (G) the same plus 4 drops wheat germ oil weekly. (J) contained 4% lard and (H) 20% lard instead of corresponding amounts of sucrose. The figures represent the number of animals whose weights were averaged.

On the diet (H) containing 20% lard the rate of early growth was definitely superior to that shown by rats receiving diet A, but growth ceased earlier and the maximum weights attained were comparable to those of control animals (diet A). The female animals on high lard diets, like the males, were slightly heavier during adolescent growth than the controls. The superior early growth of animals fed lard has also been noted by Burr ('36).

TABLE 2

Reproductive capacity and behavior

DIET	NO. OF ANIMALS TESTED	AGE IN DAYS	MALE ANIMALS, CONDITION OF TESTES				NO. OF ANIMALS TESTED	FEMALE ANIMALS, FIRST GESTATION		
			No. degenerated	No. normal	% weight of normal [1]	% fertility [2]		No. of resorptions	No. of births	No. of young per litter
A	12	130–303	12	0	45– 98	0.5–20	6	6	0	
B	5	130	0	5	106–127[3]		6	0	6	6– 9
C	6	130	0	6	113–145[3]		6	1	5	8–13
F	6	130	6	0	44– 54	3–10	5	5	0	
G	5	130	0	5	85–121	100	7	2	5	2– 9
G	4	175, 244	3	1?	51– 70	1–3, 75				
H	5	130–301	5	0	51–150	5–20	4	4	0	
J, K	9	166–175	9	0	48– 78	0.5–2[4]				
L	4	166	4	0	54– 74	1–33				

[1] Compared with Donaldson's tables. The occasional marked edema lessens the significance of this figure and also the correlation between this and the per cent fertility.

[2] Approximate per cent of normal tubules estimated by Doctor Witschi from his stained sections.

[3] Motile sperm; testes not sectioned.

[4] Five animals; in four animals, non-motile sperm, testes not sectioned.

A number of second generation animals, both male and female, born of mothers on E-deficient diets following the administration of adequate doses of vitamin E, were grown to sexual maturity on diets with and without supplements of the vitamin. The animals that had never had access to vitamin E grew, through adolescence, just as well as those receiving it, but thereafter, like the first generation, they lagged behind. They never developed symptoms of malnutrition or paresis.

The effects of these rations on reproductive capacity and behavior are summarized in table 2. The female animals on diets devoid of vitamin E demonstrated no first litter fertilities while, with two exceptions, those whose diets contained some vitamin E gave birth to litters at the first gestation. One of the exceptions was the lot receiving wheat germ oil supplement (diet G); the amount supplied seems to have been inadequate as appears also in the corresponding data from the male animals. One lot of these males, sacrificed at 130 days of age, was normal, while a second lot which lived longer showed extensive degeneration. The oil was not assayed again at the end of the experiment and the amount of vitamin E may have decreased through gradual deterioration of the oil. The male animals on the other diets demonstrated sterility or fertility in accordance with expectations.

The sharp contrast with respect to fertility between the animals on diet A, devoid of vitamin E, and diets B and C containing standardized amounts of a concentrate, together with the similarity of the early growth rates indicates that vitamin E is not required for adolescent growth. Whether the apparently earlier susceptibility of male than of female animals to the absence of vitamin E is a true expression of different requirements cannot yet be stated. The factors concerned in producing the disabilities described by Ringsted and Blumberg also remain to be determined; that there are other associated fat-soluble factors is evident from recent work on the vitamin B complex and on the dietary production of muscular dystrophy in herbivora. The existence of at least three specific substances having vitamin E activity [5] (Evans, Emerson and Emerson, '36) may clarify as well as complicate the situation.

It is improbable that the lard in the stock vitamin E-deficient diet is the source of any appreciable amount of vitamin E, even without undergoing preliminary rancidification. In the comparable instances, vitamin E concentrates were as effective in preventing resorptions in the animals on the diet (A)

[5] Personal communication from Dr. O. H. Emerson.

rigorously freed of all traces of vitamin E as in the animals on the stock E-deficient diet containing lard and unextracted casein and yeast. Statistical proof requires a much larger number of animals than were available but for practical and assay purposes a more rigorous purification of the E-deficient ration seems unnecessary. On the other hand, such a procedure is the only one which will yield information on the unknown fat-soluble factors of the diet which affect growth and well-being.

For the first time, apparently, a ration has been designed in which all of the lipid constituents are of known chemical composition, and on which animals can grow and reproduce normally. Lactation is, however, generally a complete failure despite the generous amounts of vitamin E administered. Such a ration should serve as a satisfactory basal diet for the study of the unknown factors in lactation.

SUMMARY

Rats were fed a synthetic diet, entirely free from vitamin E, containing extracted casein and yeast, sucrose, distilled ethyl esters of the fatty acids of hydrogenated cottonseed oil, carotene, calciferol and a salt mixture. The adolescent growth rate of the animals was approximately equal to that shown by animals receiving, in addition, highly purified concentrates of vitamin E in amounts adequate to produce fertility. This was also demonstrated by second generation animals which had never received vitamin E.

From the age of 2 months onward the male animals receiving vitamin E surpassed the controls in weight, and when they were sacrificed at 5 months of age they weighed approximately 10% more than the controls. There were no differences in the female animals at the age of 3 to 4 months.

The effectiveness of the vitamin E supplements was confirmed by the reproductive performance of the female animals and, in the case of the males, by the weight and histology of the testes.

Early growth in both sexes was accelerated by the inclusion of lard in vitamin E-deficient diets.

An uncomplicated vitamin E deficiency is not the cause of the early decline in growth rate and the appearance of serious malnutrition and paralysis recently observed by Blumberg and Ringsted; normal early growth is not dependent on the presence of vitamin E.

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THE RELATION OF ASCORBIC ACID INGESTION TO MINERAL METABOLISM IN CHILDREN

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From the data available (Holst and Frölich, '07; Bauman and Howard, '12, '17; Howard and Ingwaldsen, '17; Howe, '20, '22, '24; Robb et al., '21; Toverud, '23; Mellanby, '29; Michaux, '33), the connection between scurvy and mineral metabolism is not clear. If such a relationship exists, evidence of this should be obtained through mineral balance and vitamin C studies in man during the growth period. So far as we are aware, the only study with children in any way related to the subject is that of Chaney and Blunt ('25) in which the effects of the addition of orange juice on the mineral metabolism of two girls 10 and 11 years of age, respectively, were tested. The results suggest that the increased retentions following the orange juice ingestions may have been due to the vitamin C since the control diet was quite lacking in this essential substance and the retentions were far greater than could be accounted for by the increased mineral ingestions. On the other hand, orange juice may contain some substance or substances other than vitamin C which were instrumental in bringing about the higher retentions. Nor is it certain that the condition of the children was not an influencing factor. At the beginning of the study, the children showed "some indications of malnutrition, but were free to gain." Depleted children do not respond equally to increased food intakes. A 3-day or even a 5-day preliminary period may be insufficient

for metabolic adjustment depending upon the physiologic fullness of the tissues of the individuals under observation. The higher retentions of a second period immediately following the first may be due to a better diet in the first as well as in the second period. The data of short metabolism periods, which were used by Chaney and Blunt, may be misleading unless daily balances are made. With the exception of sulphur, Bauman and Howard ('12) obtained higher retentions during the first 2 days following the addition of orange juice to the diet of the patient suffering from scurvy than during the subsequent 5-day period, suggesting that these increases may have been due to previous depletion, the result of a poor diet immediately preceding the test, rather than the lack of the antiscorbutic vitamin per se.

EXPERIMENTAL PROCEDURE

A recent investigation of the vitamin C needs of children of preschool age (Everson and Daniels, '36) offered an opportunity to study the influence of various levels of vitamin C ingestion on mineral retentions; and although at no time were the diets of the children under observation entirely free from vitamin C, there were periods during which considerably less than the minimum requirement was being taken, as shown by the ascorbic acid retentions. The longer than usual metabolism periods consisting of 15 days each, 5 for physiologic adjustment to a given ascorbic acid ingestion level, and two successive 5-day collection periods, would seem to give significance to the findings, since this should have allowed time for the ascorbic acid to influence the mineral metabolism. Furthermore, during the twenty-one metabolism periods, covering 5 months of study, the food intake for a given child with the exception of orange juice or commercial ascorbic acid when used, was very constant, and insofar as known, adequate. The same amounts of the various foods for a given child were used in all periods, excepting in those cases when slight increases were made to meet the caloric needs of the children. Conditions affected by variations in vitamin D would seem to

have been ruled out by the daily ingestions of cod liver oil and viosterol, supplying approximately 3000 units (U.S.P.), the 10-minute irradiation with a mercury quartz lamp, and a playroom equipped with corex glass windows. Comparisons of mineral retentions during the various metabolism periods with the time spent out of doors indicated that this was without influence in this particular study.

The details of the study, including methods of testing the ascorbic acid intake and excretion, the preparation of the food and general care of the children are given in the former paper (Everson and Daniels). Nitrogen, phosphorus, calcium and magnesium determinations with the exception of the calcium and magnesium of the urine, were made by the methods in a previous report (Daniels et al., '35). The work was started with the idea that the urinary calcium excretions at the same levels of ingestion would be fairly comparable for a given child. The variations during the first periods led to a study of the calcium of the urines by the more frequently used method of McCrudden ('09) and that of Washburn and Shear ('32) with ashed urine. Checks of repeated tests with the latter method were found to be more nearly uniform, although in many instances results by the two methods were the same. After somewhat extensive tests, the method of Washburn and Shear became the method of choice.

Nitrogen determinations, in each case, were made with the 5-day pooled urines. The addition of 2 to 5 drops of sulphuric acid to each urine specimen for the purpose of preserving the ascorbic acid resulted in more or less acid urines; therefore, each day after these were made up to volume, enough acid was added to bring the total to a pH of 2.7 to 3. On standing, the uric acid precipitated. This was filtered off before aliquots were taken for calcium and nitrogen determinations. Thus the urinary nitrogens are slightly less than their true values and, the nitrogen retentions correspondingly higher; but since the same technics were used in all cases, the slight error introduced is the same in each balance reported.

For further study of the influence of vitamin C on mineral metabolism, we have assembled data from previous balance studies in which the ascorbic acid was obtained chiefly from weighed amounts of orange juice and canned tomatoes. The vitamin C content of these and of the other foods which contained minimal amounts was not determined directly. The figures used are estimates based on the averages of the determinations in the balance study, or as with tomatoes, a subsequent determination. We appreciate this introduces some error, but not enough, we believe, to invalidate the results, especially since the majority of the ascorbic acid ingestions were found to be considerably in excess of the minimum requirement.

These selected studies have differed from those of the ascorbic acid balance study in that different amounts of calcium, from 1 quart or 1 pint of milk, respectively, were taken as well as different amounts of orange juice. It seemed possible that the influence of ascorbic acid on calcium retentions might be more obvious at lower levels of calcium ingestion. Similarly, the amount of orange juice as such, 60 cc. and 120 cc., may have influenced the retentions. The children studied were of the same age, and during the metabolism periods lived under the same controlled conditions for the same length of time as did those of the ascorbic acid balance study. Cod liver oil and viosterol also were given daily, the same amounts and brands being used in all cases. The orange juice was divided into two parts, one portion being given at 10 A.M., and the other between 3.30 and 4 P.M.

RESULTS

The findings of the ascorbic acid balance study (Everson and Daniels) indicate that under the conditions of the study optimum retentions of ascorbic acid are obtained in children of the age considered (39 to 59 months) when from 6 to 7.5 mg. per kilogram are included in the diet. Ingestions of greater magnitude resulted in no higher retentions. If mineral retentions, more particularly calcium, phosphorus and magnesium are influenced by ascorbic acid ingestions, it would

seem that low values should be obtained when the intakes fall below 6 mg., and consistently high retentions with ingestions above 7.5 mg. at a given calcium ingestion level.

The mineral retentions of the children receiving the constant diets which varied only in the amounts of orange juice and vitamin C suggest that there is no direct relation between ascorbic acid retention and calcium, phosphorus and magnesium retention within the limits of the study. With the exception of nitrogen the average retentions with ascorbic acid ingestions below 4.5 mg. per kilogram were the same as those during ingestions of 6.7 to 11.5 mg. per kilogram (table 1). This lack of relationship between ascorbic acid at the levels tested and mineral metabolism is further indicated by the results with both the higher and lower levels of mineral, more particularly calcium, ingestion (table 2). The highest calcium value obtained, 17.5 mg. per kilogram occurred during an ascorbic acid intake of 7.2 mg. per kilogram with 80 mg. of calcium per kilogram, whereas 13.5 mg. were retained during an ingestion of 8.3 mg. of ascorbic acid and 51 mg. of calcium. Ascorbic acid ingestions of twice the physiologic minimum resulted in no higher calcium retentions than when just enough to cover the minimum requirement was being taken.

That orange juice as such in the amounts tested has no influence on mineral retentions when included in a diet which contains insofar as is known adequate amounts of all the known essential constituents is shown both by the ascorbic acid balance studies and the controlled ascorbic acid studies. The minimum orange juice tested was 30 cc., the optimum 120 cc. At these two levels of ingestion, calcium and phosphorus retentions were practically the same (table 1) nor were the retentions in the controlled balance study significantly different at the various levels of ingestion (table 2).

The most suggestive findings of the ascorbic acid balance study are concerned with the nitrogen metabolism. The average retention during the period of low intake is 27.1% less than that of the higher level (table 1). These lower retentions of the first group are the more significant since of these, three

TABLE 1

Influence of ascorbic acid ingestion on the mineral metabolism of children receiving constant diets

NUMBER OF PERIODS	AVERAGE WEIGHT	INGESTION							RETENTION PER KILOGRAM										AVERAGE OUTDOOR PLAY PER DAY
				Average per kilogram					Ascorbic acid		Calcium		Phosphorus		Nitrogen		Magnesium		
		Orange juice	Redoxon [1]	Ascorbic acid	Calcium	Phosphorus	Nitrogen	Magnesium	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	
	kg.	*cc.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*min.*
3 [2]	18.2	30	..	2.8	64	67	513	13.5	2.1	2.0–2.4	9.2	6.9–10.6	5.2	3.9–5.9	36	21–43	0.6	0.4–0.8	21
3 [2]	18.7	60	..	3.7	63	71	553	14.6	2.3	1.9–2.6	10.0	8.7–11.3	6.2	4.7–8.6	41	20–62	1.1	0.6–1.4	61
3 [2]	17.9	60	..	3.9	66	72	556	14.7	2.7	2.6–2.7	9.1	7.0–10.8	6.3	5.4–7.2	55	53–58	0.9	0.7–1.0	60
3 [2]	15.6	60	..	4.4	72	75	550	15.3	3.0	2.8–3.2	11.2	7.9–14.7	6.3	5.5–7.1	40	33–46	1.0	0.7–1.3	60
Average				*3.7*	*66*	*71*	*543*	*14.5*	*2.5*		*9.9*		*6.0*		*43*		*0.9*		*51*
3 [2]	16.9	60	48	6.7	68	74	560	14.8	3.3	3.0–3.7	11.2	10.3–12.4	5.4	4.5–6.1	56	42–64	0.5	0 –1.0	80
3 [2]	17.4	120	..	6.9	67	73	550	15.3	3.9	3.6–4.2	8.9	6.9–10.2	5.7	5.4–6.2	55	49–59	1.1	0.9–1.3	36
3 [2]	18.3	120	96	11.5	65	69	530	14.3	3.5	3.2–4.0	10.3	6.2–15.5	6.2	5.8–6.7	65	51–86	1.1	0.9–1.4	0
Average				*8.4*	*67*	*72*	*546*	*14.8*	*3.6*		*10.1*		*5.8*		*59*		*0.9*		*39*

[1] Commercial ascorbic acid—Hoffman-La Roche Co.

[2] Averages are the results obtained with the three children studied.

[3] In each case, the averages are the results obtained with a given child.

TABLE 2

Relation of ascorbic acid ingestion to mineral retentions of children receiving diets containing two levels of milk

NUMBER OF CASES	AVERAGE WEIGHT	INGESTION PER KILOGRAM				RETENTION PER KILOGRAM					
		Ascorbic acid, average	Calcium, average	Phosphorus, average	Nitrogen, average	Calcium		Phosphorus		Nitrogen	
						Average	Range	Average	Range	Average	Range
Diets containing 486 gm. milk											
	kg.	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*	*mg.*
1	14.3	12.2[1]	54	76	570	8.5	.....	6.2	.....	80	.....
4	15.6	11.4[1]	53	69	562	10.2	6.5–12.4	7.7	4.5–10.0	90	56–120
2	16.2	10.4[1]	51	65	550	10.1	9.9–10.4	5.9	4.0– 7.9	78	78– 78
2	14.4	9.2[2]	56	64	510	6.1	5.8– 6.5	6.0	5.6– 6.4	83	57–110
5	16.0	8.3[2]	51	63	490	10.3	6.9–13.5	9.3	7.7–11.3	86	77– 96
5	17.1	7.2[2]	48	61	478	8.2	6.0–10.1	5.4	2.9– 6.7	60	38– 77
3	19.6	6.4[2]	44	56	450	8.6	7.4– 9.9	4.6	3.3– 5.8	41	39– 47
1	19.9	5.9[2]	42	56	470	6.5	.....	1.8	.....	41	.....
Diets containing 976 gm. milk											
1	14.7	12.3[1]	90	90	580	14.8	.....	5.0	.....	67	.....
2	15.8	11.3[1]	80	81	550	10.3	9.9–10.7	6.1	4.5– 7.7	84	76– 92
1	16.6	10.6[1]	81	78	540	11.2	.....	8.5	.....	98	.....
1	14.0	9.1[2]	98	84	570	11.1	.....	15.7	.....	75	.....
3	14.8	8.6[2]	88	83	570	12.1	9.4–14.8	6.8	4.4–10.0	68	50– 91
4	16.9	7.2[2]	80	78	570	10.6	7.8–17.5	6.5	4.9– 8.3	60	50– 76
4	19.6	6.5[2]	72	75	555	8.8	6.9–10.3	4.9	3.5– 7.6	50	46– 54

[1] Diets included 120 cc. orange juice.

[2] Diets included 60 cc. orange juice.

balance studies were the first metabolism periods of the series and three other periods immediately following the 3-week Christmas holiday—periods when it is not unusual to obtain high retentions which we have attributed to the less well controlled home diets of the children previous to entrance to the metabolism ward. Because of past experience, neither the highest nor lowest ascorbic acid ingestions were tested at these times. Nitrogen retentions in the balance studies in which the ascorbic acid ingestions were estimated (table 2) indicate that with few exceptions sufficient ascorbic acid had been taken. The average nitrogen retentions are not consistently related to ascorbic acid ingestion. The range of retentions, furthermore, shows that high retentions may be obtained at any level of ingestion above 6.4 mg. per kilogram, or thereabout. The low retentions at the lowest ingestion levels are in line with the findings of the ascorbic acid balance study.

COMMENTS

In evaluating the effect of orange juice on mineral retentions, it should be noted that in no case was all of the ascorbic acid obtained from orange juice. Given amounts of raw banana were included in all diets and with the exception of the ascorbic acid balance study, all diets contained some canned tomatoes. In diets containing all the known essential substances in seemingly adequate amounts, orange juice apparently is without influence on the calcium, phosphorus and magnesium retention. With other types of diets, orange juice may contribute some substance or substances essential to mineral metabolism, which in our diets were obtained from other foods. Without more knowledge, one hesitates to hazard a conjecture as to what substance beside vitamin C was below the physiological minimum in the Chaney and Blunt diets. Their basal diets were neutral, changing to basic with the addition of orange juice, whereas those used for the ascorbic acid studies were basic at all levels of orange juice ingestion. Vitamin D, in the Chaney and Blunt study was uncontrolled and not given as such, and although "the children led a regular

happy life with plenty of out-door sunshine," more out-of-doors may have been available during the orange juice periods, thus accounting for the higher retentions during that period; or, as has been suggested, the increased retentions may have been due to a stimulation of growth resulting from the better diets of both periods, the effects being more evident in the second. There seems to be no evidence either in the studies herein reported, or in those of Bauman and Howard to support the thesis that in man vitamin C is directly concerned with calcium and magnesium metabolism. Indirectly, when not present in sufficient amounts over considerable periods, it may be related by decreasing the oxidative capacity of the tissues (Harrison, '33) and by bringing about such profound tissue changes (Howe, '20; Wolbach and Howe, '26; Menkin, Wolbach and Menkin, '33; Siehrs and Miller, '34; Park et al., '35) that these essential mineral substances cannot be fixed.

In line with these tissue changes are the findings of Bauman and Howard ('12), Howard and Ingwaldsen ('17), and Kachevnik and Eidman ('35), suggesting that scurvy is a disease primarily affecting nitrogen metabolism. With the patient suffering from scurvy, nitrogen retentions which were negative when the patient was receiving a diet containing no vitamin C became positive when orange juice was added to the diet. With the monkey and the guinea pigs in which scurvy was developed, the nitrogen excretions increased as the disease progressed. Chaney and Blunt found higher nitrogen retentions in the children studied when orange juice was added to the diet. The lower nitrogen retentions of the children receiving less than the physiological minimum of vitamin C in the ascorbic acid balance study corroborate these findings and offer an explanation for those studies in which growth was stimulated by the addition of orange juice to the diets of underweight children (Chaney, '23; Morgan et al., '26).

We began the study anticipating that at low vitamin C levels, calcium metabolism might be affected, therefore the results were the more surprising, as were the findings of the influence on nitrogen retentions of vitamin C at ingestion levels

which we have hitherto believed to be adequate. It is not customary for children to be given as much vitamin C as the ascorbic acid balance study indicates they should have, one raw fruit a day being the usual dietary dictum. One is led to consider what proportion of children are suffering from undernutrition because of too little vitamin C of the diet and what the ultimate effects of less than the physiological requirement over a considerable period will be. Studies with guinea pigs have seemed to point to a relation between vitamin C, calcium metabolism and dental caries. In man, this relationship has not been established. In view of our results, it seems possible that a continued ingestion of less than the optimum amount of vitamin C may so affect the dental tissues that calcium will fail to be retained. Further work is needed to confirm this hypothesis.

SUMMARY

Calcium, phosphorus, magnesium and nitrogen retentions in children of preschool age have been studied in relation to ascorbic acid ingestions and retentions during periods when the children were receiving diets which included 1 quart, 3 cups and 1 pint of milk, respectively. The influence of two levels of orange juice ingestion, 60 cc. and 120 cc., also has been considered. Variation in vitamin D as an influencing factor was seemingly ruled out since each child received approximately 3000 U. S. P. units per day. Each child, furthermore, was given a 10-minute exposure to ultra violet light from a mercury quartz lamp and lived in a room equipped with corex glass windows. The time spent out of doors showed no correlation with the retentions.

The findings indicate that within the limits of the study:

1. Ascorbic acid ingestions between 2.7 and 12.5 mg. per kilogram have no influence on calcium, phosphorus and magnesium retentions. In the controlled ascorbic acid balance study in which the ascorbic acid retentions were shown to be below the physiologic minimum, there was found to be no appreciable difference in the average retentions of calcium, phosphorus

and magnesium. Individual differences noted were not related to the ascorbic acid ingestions.

2. There is no apparent relationship between the calcium and phosphorus retentions and the calcium and ascorbic acid of the diet when more than the physiological minimum of either is taken.

3. Ascorbic acid seems to be related to nitrogen metabolism. The average nitrogen retentions at levels of ascorbic acid ingestions below the physiological minimum were found to be lower than when the children were receiving adequate amounts as shown by the ascorbic acid retentions. Ascorbic acid ingestions in excess of the physiologic requirement, however, do not further increase the nitrogen retention.

4. Orange juice per se, at the levels tested, 60 cc. and 120 cc. per day, when given in conjunction with diets containing insofar as is known all the essential constituents in adequate amounts, is without influence on the calcium, phosphorus, magnesium and nitrogen retentions.

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